The investigation of chemokine-mediated signalling, regulation and receptor recycling in lymphocytes

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Award date:
2004

Awarding institution:
University of Bath

THE INVESTIGATION OF CHEMOKINE-MEDIATED SIGNALLING, REGULATION AND RECEPTOR RECYCLING IN LYMPHOCYTES

submitted by

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for the degree of PhD

of the University of Bath

2004

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In memory of Gemma Sharp

Acknowledgements

I, Bob, would like to show my appreciation to my supervisor Dr Stephen Ward for his support and guidance throughout. Also, a big thank you goes to my industrial supervisor Professor John Westwick of Novartis Pharmaceuticals for his support and generosity.

My fellow Wardettes……I love you all. Boys you have educated me with music, pool, lab rounders and bomb construction…….I thank you, I think! Girls what would I have done without you. Karj (otherwise known as Bird), words cannot express my gratitude for your friendship; you are one in a million. I would particularly like to thank you for your hospitality and for getting me through the difficult periods of my thesis write up.

Others that should not go un-mentioned include: Dr Zarin Brown, Jenny Graham and Chris Rothwell of Novartis Pharmaceuticals; Professor Ann Richmond and Assistant Professor Guo-Huang Fan of Vanderbilt University, Nashville USA, who were all very helpful and kind during my placements.

One special person who is such an inspiration in my life is my dearest friend Gemma Sharp, who sadly lost her life at the age of 19. I dedicate this thesis in memory of you.

Finally and most importantly, I would like to thank my family. Yes Sarah, that includes you! My parents have been a rock, bringing me up to believe that hard work will always pay off in the end. They have shown me so much love and support despite all that I have thrown their way, and I will always be in their debt for this. I may be off to Boston but you will always be close in my heart.

Abstract

The research within this thesis primarily concentrates on the two chemokine receptors CCR8 and CXCR4. The chemokine family is divided into 4 groups, where CCR8 and CXCR4 fall into each of the two main subfamilies. The first section focuses on the signalling pathways mediated by the CCL1/CCR8 chemokine/chemokine receptor pair. CCL1 is described as a potent Th2 lymphocyte chemoattractant and is therefore under investigation as a potential treatment for asthma. Thus, examination of the ambiguous biochemical and functional pathways elicited by CCL1 would provide a novel insight for future drug developments. This study demonstrated for the first time that ligation of CCR8 leads to the activation of both the ERK1/2 MAPK and PI3K/PKB pathways. In addition, the CCL1-stimulated migration of a leukaemic T cell line exhibited classic bell-shaped, concentration-dependent characteristics, a phenomenon widely associated with chemokine receptors in general. Taken together, these data show that CCR8-mediated responses appear to be analogous with that of other well-defined chemokine receptors.

The following 2 sections examined the chemokine receptor CXCR4 and its ligand CXCL12. CXCR4 is fundamental in organ development and has been identified as a co-receptor for HIV. The first section aimed to determine the role of the inositol-lipid phosphatase SHIP in the inhibitoy regulation of chemokine signalling. Investigation into the negative regulation of chemokine-mediated signalling pathways by SHIP is ill-defined. Failure to initiate inhibitory feedback mechanisms results in profound immune defects, as seen in autoimmunity. This study demonstrates in B cells that co-ligation of the BCR and FcγRIIb receptor can not only attenuate BCR-mediated responses but also those induced by chemokine receptors. In addition, a T cell model that expresses a tetracycline-regulated, constitutively active SHIP mutant showed that SHIP can impinge on CXCL12-induced PI(3,4,5)P_3 levels, PKB phosphorylation and chemotaxis. Thus, these data implicate a regulatory role for SHIP in chemokine-mediated responses.

The concluding section investigates the role of the class II PI3K-C2β in chemokine receptor recycling, which has been shown to be essential for chemokine-mediated cell migration. In summary, these data suggest that class II PI3Ks may co-localise with CXCR4 and AP2 and hence contribute to the local phosphoinositide levels that play a central role in receptor sequestration.

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Abbreviations

Ab	Antibody
ADP	Adenosine di-phosphate
Ag	Antigen
APS	Ammonium Persulphate
AP	Adaptor Protein/ Adaptin
βARK1	Beta Adrenergic Receptor Kinase 1
ATP	Adenosine tri-phosphate
BSA	Bovine Serum Albumin
BCR	B cell receptor
Ca^{2+}	Calcium Ion
CALM	Clathrin Assembly Lymphoid Myeloid Leukaemia Protein
cAMP	Cyclic adenosine monophosphate
CCV	Clathrin Coated Vesicle
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGTA	Ethyleneglycol-bis(β-amino-ethylether)-N, N, N',N'tetraacetic acid
ER	Endoplasmic Reticulum

ERK1/2	Extracellular Signal Regulated Kinase 1/2
FBS	Foetal Bovine Serum
FITC	Fluorescein isothiocyanate
GEF	Guanine Exchange Factor
GFP	Green Fluorescent Protein
GPCR	G protein-coupled Receptor
G-protein	GTP-binding protein
GRK	G-protein-related kinase
GTP	Guanosine trisphosphate
GST	Glutathione S-Transferase
GTP	Guanosine trisphosphate
HBSS	Hank's balanced salt solution
HEPES	N-[2-hydroxyethyl]piperazine-N'[2-ethanesulphonic acid]
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
hr	Human Recombinant
ICAM	Intercellular adhesion molecule
IgG/M	Immunoglobulin G /M
IFN	Interferon
IP	Immunoprecipitate
IP_3	Inositol trisphosphate
IP_3R	IP_3 Receptor
IκB	Inhibitor of NFκB

IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
kDa	Kilodalton
MAPK	Mitogen activated protein kinase
mAb	Monoclonal antibody
MLC	Myosin Light Chain Kinase
MLCK	Myosin Light Chain
MEK	MAPK kinase
mRNA	Messenger ribonucleic acid
M-tropic HIV	Macrophage tropic HIV
NFκB	Nuclear factor kappa-B
NK	Natural killer
PAK	p21-activated kinase
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
PDGF	Platelet derived growth factor
PDK-1	Phosphoinositide dependent kinase-1
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PI/PIP/PIP_2	Phosphoinositide/mono/bis phosphate
PIP3/$PI(3,4,5)P_3$	Phosphoinositide(3,4,5)trisphosphate
PKB/PKC	Protein kinase B (Akt)/C

PLC	Phospholipase C
PMA	Phorbol-12-myristate-13-acetate
PNRC	Perinuclear Recycling Compartment
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
PTK	Protein tyrosine kinase
PTX	Pertussis toxin
PVPF	Polyvinylpyrrolidine free
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SEB	Staphylococcal enterotoxin B
SEM	Standard error of means
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH	Src homology
SHP	Src homology 2-Containing Phosphatase
SHIP	SH2-containing inositol 5' polyphosphatase
STAT	Signal transducer and activator of transcription
TBS	Tris buffered saline
TCR	T cell Receptor
TEMED	N,N,N',N'-tetramethylethylendiamine
TGN	Trans-Golgi Network
Th	T helper
TLC	Thin layer chromatography

Chapter 1

Chapter 1: Introduction

1.1 Introduction

Innate and adaptive immunity constitute a defence system that protects an organism from attack. In the event of tissue damage or invasion by foreign bodies, the innate response of vascularized tissue, known as inflammation, is activated. This process is highly reliant on the inherent aptitude of leukocytes to gain access to the affected areas. An additional event required for the induction of cellular immunity is for dendritic cells to migrate from the infected peripheral tissues to the draining lymphoid organ, bearing antigens from the infected agent (1). Upon arrival at the T cell area of draining lymph nodes, dendritic cells interact with rare antigen specific lymphocytes. The induction of T cell division by antigen-loaded dendritic cells is very rapid, even within 2 days some antigen specific T cells have already divided twice. A further 24-48 hours later, divided cells have acquired effector phenotype and migrate from the draining node to the periphery (2).

The adaptive immune system protects the host from succumbing to pathogens previously encountered. Antigen-specific memory lymphocytes generated during an initial infection or by vaccine administration, become rapidly mobilized to elicit a potent recall immune response to direct pathogen clearance (3).

Each day the body produces billions of new leukocytes to replace those lost to normal cell turnover processes as well as to illness or trauma. This process known as haematopoiesis is dependent on migratory stimuli to guide differentiated progeny to sites where they can undergo correct development.

Thus, without doubt, an essential component of the immune system is the homing and control of cellular localisation. In its absence, the fate and function of leukocytes is abrogated and the defence system of the host fails. Moreover, inappropriate activation of an inflammatory response leads to diseases such as atherosclerosis, arthritis, asthma and psoriasis. So how is leukocyte localisation regulated? Molecules expressed on the vascular endothelium are known to be involved in the regulation of leukocyte extravasation and include; selectins; integrins; cadherins; connexins and chemoattractants (prostaglandins, C5a, formyl peptide) (4) however it is without question that the chemokine family of secreted proteins play a central role in this process (5-9).

1.2 Chemokines

Chemokines are a superfamily of low molecular weight proteins that include more than 40 members in humans (10). They were originally named according to their function by the scientists who discovered them. However, this system began to fail following the concurrent identification of the same chemokine by different groups.

Consequently, the Nomenclature Committee of the International Union of Pharmacology (NC-IUPHAR) led by Yoshie and Zlotnick was formed to develop a novel nomenclature system which was based on protein structure and a previous nomenclature for chemokine gene loci (11) (Table 1).

CXC Chemokine/Receptor Family

Systematic Name	Human Chromosome	Human Ligand	Chemokine Receptor(s)
CXCL1	4q12-q13	GROα/MGSA-α	CXCR2 >
CXCL2	4q12-q13	GROβ/MGSA-β	CXCR1
CXCL3	4q12-q13	GROγ/MGSA-γ	CXCR2
CXCL4	4q12-q13	PF4	CXCR2
CXCL5	4q12-q13	ENA-78	Unknown
CXCL6	4q12-q13	GCP-2	CXCR2
CXCL7	4q12-q13	NAP-2	CXCR1, CXCR2
CXCL8	4q12-q13	IL-8	CXCR2
CXCL9	4q21-21	Mig	CXCR1, CXCR2
CXCL10	4q21-21	IP-10	CXCR3
CXCL11	4q21-21	I-TAC	CXCR3
CXCL12	10q11.1	SDF-1α/β	CXCR3
CXCL13	4q21	BLC/BCA-1	CXCR4
CXCL14	Unknown	BRAK/bolekine	CXCR5
CXCL15	Unknown	Unknown (Lungkine in mouse)	Unknown Unknown

C Chemokine/Receptor Family

Systematic Name	Human Chromosome	Human Ligand	Chemokine Receptor(s)
XCL1	1q23	Lymphotactin	XCR1
XCL2	1q23	SCM-1b	XCR1

CX3C Chemokine/Receptor Family

Systematic Name	Human Chromosome	Human Ligand	Chemokine Receptor(s)
CX3CL1	16q13	Fractalkine	CX3CR1

CC Chemokine/Receptor Family

Systematic Name	Human Chromosome	Human Ligand	Chemokine Receptor(s)
CCL1	17q11.2	I-309	CCR8
CCL2	17q11.2	MCP-1/MCAF	CCR2
CCL3	17q11.2	MIP-1α/LD78α	CCR1, CCR5
CCL4	17q11.2	MIP1-β	CCR5
CCL5	17q11.2	RANTES	CCR1, CCR3,
(CCL6)		Unknown	CCR5
		(mouse only)	Unknown
CCL7	17q11.2	MCP-3	CCR1, CCR2,
CCL8	17q11.2	MCP-2	CCR3
(CCL9/CCL10)		Unknown	CCR3
		(mouse only)	Unknown
CCL11	17q11.2	Eotaxin	CCR3
(CCL12)		Unknown	Unknown
		(mouse only)	CCR2, CCR3
CCL13	17q11.2	MCP-4	CCR1
CCL14	17q11.2	HCC-1	CCR1, CCR3
CCL15	17q11.2	HCC-2/Lkn-1/MIP-1δ	CCR1
CCL16	17q11.2	HCC-4/LEC	CCR4
CCL17	16q13	TARC	Unknown
CCL18	17q11.2	DC-CK1/PARC/AMAC-1	CCR7
CCL19	9q13	MIP-3β/ELC/exodus-3	CCR6
CCL20	2q33-q37	MIP-3α/SLC/exodus-1	CCR7
CCL21	9q13	6Ckine/SLC/exodus-2	CCR4
CCL22	16q13	MDC/STCP-1	CCR1
CCL23	17q11.2	MPIF-1	CCR3
CCL24	7q11.23	MPIF-2/Eotaxin-2	CCR9
CCL25	19q13.2	TECK	CCR3
CCL26	7q11.23	Eotaxin-3	CCR10
CCL27	9q13	CTACK/ILC	

Table 1. The Chemokine Family

The new nomenclature system developed by A. Zlotnick and O.Yoshie in 2000. This table also includes the old nomenclature system and highlights the clusters of chemokines that are found on chromosome 4 (CXC) and 17 (CC).

Classification of Chemokines

The chemokine family is divided into two major classes, CC (β) and CXC (α), dependent upon the location of two highly conserved N–terminal cysteine residues, adjacent and separated by an intervening residue, respectively (12). The two minor families C (γ) and CX_3C (δ) chemokines are at present constituted by the members XCL1 and 2 (lymphotactin α and β) and CX_3CL1 (fractalkine), respectively. The 'C' family lack the first and the third cysteine of the typical chemokine structure (13), while CX_3C chemokines contain three amino acids between the first two cysteines and is also the only membrane-bound chemokine, and contains a mucin-like stalk (14).

ELR Chemokines

CXC chemokines are further classified according to the presence of the tripeptide motif glutamic acid-leucine-arginine (ELR) N-terminal to the first cysteine. ELR-positive chemokines are known to function as potent angiogenic factors, able to stimulate endothelial cell chemotaxis. In contrast, ELR-negative chemokines are strong angiostatic factors, which inhibit endothelial cell chemotaxis induced by ELR-positive chemokines (15). ELR-positive chemokines predominantly bind to CXCR2 with the exception of CXCL8/IL-8 which also binds CXCR1. ELR-negative chemokines bind to CXCR3, CXCR4 and CXCR5 (Table 2).

Chemokines that act predominantly on T cells

A more useful classification of chemokines, more so of the CC family, distinguishes between molecules that are made in a tonic way (constitutive) and those that are made in response to diverse signals (inducible or inflammatory) (11,16) (Figure 1). The former are those constitutively expressed in a certain tissue or organ, suggesting a specific function involving cell migration. The inflammatory chemokines in contrast are strongly upregulated by inflammatory or immune stimuli in various cell types (macrophages, fibroblasts, T cells, etc.) and are thus likely to participate in the development of immune or inflammatory reactions.

Genes encoding inflammatory chemokines are typically found in two major clusters on chromosomes 4 (CXC) and 17 (CC), whereas genes for homeostatic chemokines are located alone or in small clusters on chromosomes 1,2,5,7,9,10 and 16 (Table 1). This diversification likely reflects functional specialization that has developed during the evolution of this superfamily and suggests that those chemokines located in different chromosomal locations from the major clusters are probably older in evolutionary terms and have remained more conserved between species because of their specific functions. They are also less likely to share receptors or have overlapping functions with other chemokines. In contrast, the major cluster chemokines are likely to have been generated more recently in evolutionary terms.

The fact that the genes encoding these chemokines were allowed by evolution to duplicate rapidly and form a cluster, suggests that their functions remained to a certain extent related. The functional specificities may therefore be less important for this type of chemokine; their main function is likely to be the attraction of neutrophils or monocytes, while their specificity may be a secondary function (11).

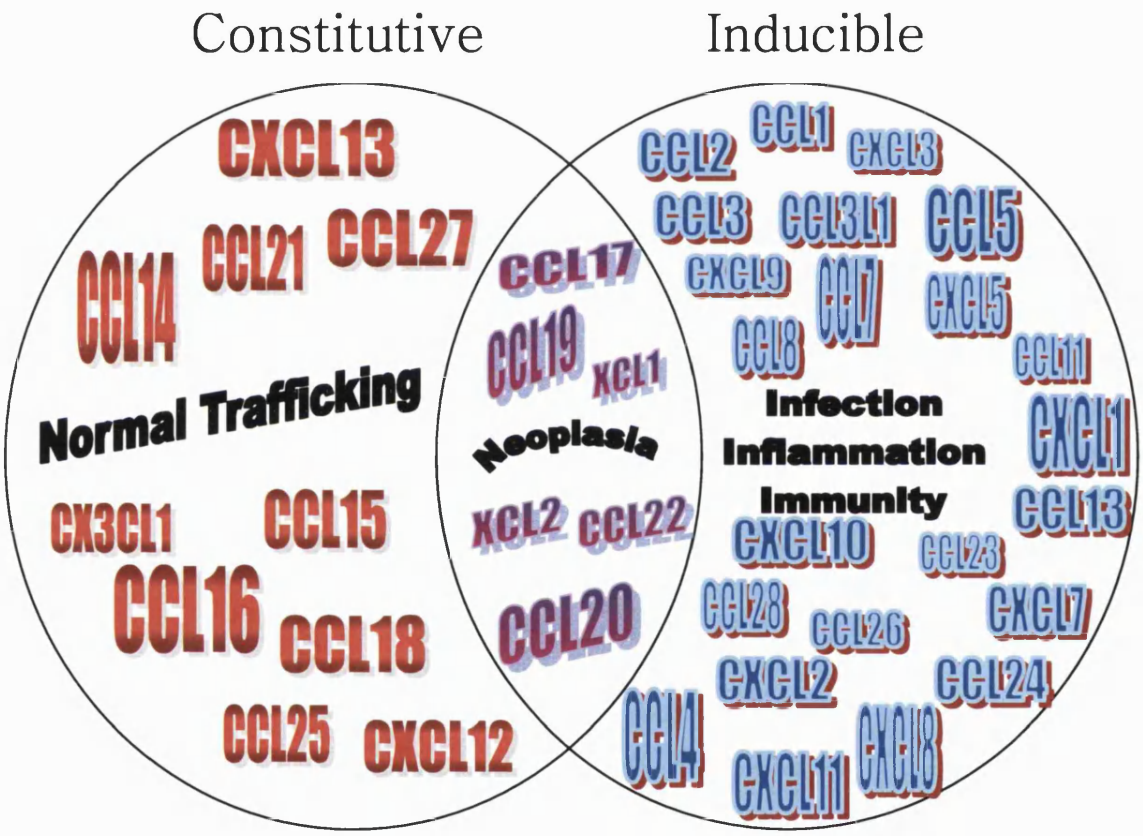

Figure 1. Functional classification of chemokines

According to the regulation of their production, chemokines can be classified as constitutive chemokines which regulate normal leukocyte trafficking or inducible chemokines that are associated with inflammatory and immune responses. However, some chemokines play a pivotal role in homeostasis as well as in inflammatory responses.

Constitutive/Homeostatic Chemokines

Constitutively expressed CC chemokines are produced in discrete microenvironments within lymphoid or non-lymphoid tissues and in general function to guide the normal traffic of leukocytes under normal conditions. For example, CCL17/TARC and CCL27/SLC control cutaneous memory T cell homing to the skin. In addition CCL21/6Ckine and CCL19/ELC help control lymphocyte entry into secondary lymphoid tissues (17,18).

CXCL12/SDF-1 and CXCR4

The 'homeostatic' chemokine CXCL12/SDF-1 falls into the CXC (α) family and has one receptor, known as CXCR4 (Table 1). The chemokine receptor CXCR4 was cloned in 1994 by Loetscher *et al* (19). It has since been found to be widely expressed on haematopoietic cell types including neutrophils, monocytes, T and B lymphocytes, B cell precursors, CD34+ progenitor cells from blood and bone marrow, blood derived dendritic cells, Langerhans cells, mature and immature T cells of the thymus, and macrophages. CXCR4 is also expressed on non-haematopoietic cells which include vascular endothelial cells, neurons of the central and peripheral nervous system, microglia and astrocytes (20).

With respect to function, CXCR4 was identified in 1996 along with CCR5 as a co-receptor for the T-tropic human immunodeficiency virus type I (HIV-I) (21), this led to a sudden surge of interest and subsequent growth in CXCR4 research. Mice deficient in either the CXCL12/SDF-1 or CXCR4 gene show major defects in cerebella development; cardiac septum formation; B cell lymphopoiesis; and gastrointestinal vascular development (Figure 2).

Inducible Chemokines

The role of inducible chemokines is to regulate the recruitment of leukocytes on demand, in response to immunological, inflammatory and infectious signals (1,22). Unfortunately, this functional division also has its limitations in terms of molecules and pathology. A number of molecules behave as both constitutive and inducible chemokines. For example, CCL19/ELC is not only constitutively expressed by stromal cells where it functions to guide naïve T cells and maturing DC into the T zones of secondary lymphoid organs, but is also upregulated along with CCL21/SLC at sites of chronic inflammation (23). Similarly, CCL22/MDC was initially described as a chemokine constitutively expressed in certain cell types, most notably dendritic cells and in certain lymphoid organs, in particular the thymus (24). Subsequent work, prompted by the recognition that this molecule attracted preferentially polarized type 2 T cells, has shown that CCL22/MDC is also expressed in a regulated way.

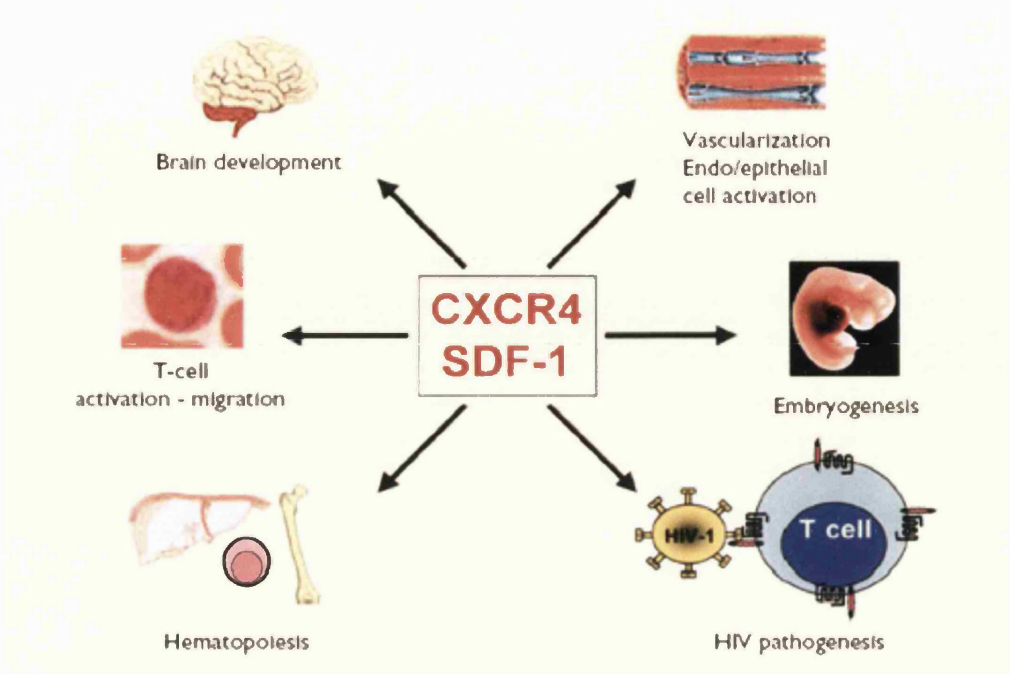

Figure 2. Summary of CXCR4/CXC12 involvement in Human Physiology

Evidence suggests that CXCR4 and CXCL12/SDF-1 are involved in brain development, vascularization, embryogenesis, T-cell activation, migration, haematopoiesis and HIV pathogenesis (25).

CCR8 and its ligand CCL1

The 'inflammatory' chemokine receptor CCR8 is a member of the CC (β) family of chemokines, and is activated following the binding of the potent chemoattractant CCL1 (Table 1) (26). In the old nomenclature system, CCL1 was called I-309 according to the identification number of its cDNA clone (27). As detailed previously, the new nomenclature system was proposed to overcome the many

chemokine aliases that had accompanied the co-discovery of chemokines by multiple groups (10). In addition to CCL1, only virally encoded chemokines exhibit a high affinity for the CCR8 receptor; HHV8 encoded vMIP-I-like CCL1 is a specific agonist for CCR8, whereas HHV8 encoded vMIP-II and MCV encoded vMCC-I act as potent antagonists, binding to CCR8 without inducing signalling and blocking the effects of CCL1 and vMIP-I (28-30).

The tissue expression of CCR8 is unique, with constitutive mRNA expression occurring in the thymus (26,31). In the periphery, however, CCR8 is found to be expressed on Th2-polarized cells (32), IL-2-activated natural killer cells (33), T_{reg} cells (34-36), endothelial cells (37,38) and eosinophils (39).

CCR8 has received considerable attention concerning its potential role in the pathogenesis of inflammation; however, despite the generation of several knockout models, the precise function remains unclear. Two studies on CCR8-deficient mice, based on an ovalbumin-induced model of asthma, reported to have no effect on the development of pulmonary eosinophilia and T helper type 2 (Th2) cytokine responses and concluded that CCR8 does not play an essential role in the pathogenesis of inflammation (40,41). However, a different group showed that mice genetically deficient in CCR8 had impaired Th2 cytokine production and eosinophil recruitment during Th2-mediated responses, with no abrogation of Th1-mediated responses (42). Bishop *et al.* subsequently demonstrated that CCL1 was up-regulated in the lung following allergen challenge. Moreover, a neutralising antibody to CCL1 was shown

to markedly reduce eosinophil migration, but have no effect on the recruitment of Th2 cells to the lung following allergen challenge. Interestingly, there was also no change in airway hyperresponsiveness or levels of Th2 cytokines (43).

There is also some evidence to support the notion that CCR8 may function as a co-receptor for HIV-1 infection (44-46), regulate the trafficking of T_{reg} and memory T cells (47-49) and modulate endothelial cell function (50). In addition, one study links CCL1 to apoptosis, where it is thought to protect lymphomas against corticoid- and dexamethasome-induced apoptosis (51-53). In addition, CCL1 is thought to play a role in monocyte chemotaxis (54) and angiogenesis (37).

1.3 Chemokine Receptors

Chemokines interact with their target cells through heptahelical cell surface G protein-coupled receptors derived from the rhodopsin family (12,55,56). These receptors, known as chemokine receptors, are typically 340-370 amino acids in length and contain the conserved DRYLAIV amino acid sequence in the second intracellular loop. The DRYLAIV motif is known to be involved in G-protein coupling, exposed following activation of the chemokine receptor by ligand binding (10,20).

In general, chemokine receptors fall into two categories of expression: those expressed exclusively on specific leukocyte subsets; and those that are more broadly

expressed. The most widely expressed chemokine receptor is CXCR4, which is present on neutrophils, monocytes, T lymphocytes, B lymphocytes and blood-derived dendritic cells amongst others. CXCR1 and CXCR2 are expressed on most leukocytes, although appear to be only functionally significant for neutrophils, monocytes/macrophages and mast cells (10,57). CXCR3, CXCR5 and CXCR6 are exclusively expressed on lymphocytes (58-60). CCR1, CCR2, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9 and CCR10 are expressed mainly on lymphocytes, monocytes and monocyte-derived dendritic cells (10). CCR3 has a unique expression pattern, as it is found on eosinophils, mast cells, basophils, Th2 lymphocytes, and certain dendritic cell populations (61,62).

A number of chemokine receptors are promiscuous in that they bind more than one chemokine, but not those outside of their class. For example, the chemokine receptor CCR5 binds CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, CCL8/MCP-2 and CCL14/HCC-1, but does not bind 'CXC', 'C' or 'CX_3C' chemokines (Table 1). The exact purpose why one chemokine binds many receptors may reflect their ability to regulate many different leukocyte subpopulations, especially in complex micro-environments such as acute or chronic inflammatory responses.

One chemokine receptor that does not meet the criteria for the systematic nomenclature is 'duffy antigen receptor for chemokines' (DARC), a seven-transmembrane domain (7TMD) binding protein that appears not to signal. DARC

also known as D6 is a highly promiscuous CC chemokine receptor, binding the majority of members of the CC chemokine family. However, it is specific for this family and shows no detectable affinity for members of the CXC, C or CX_3C chemokines (63-65).

The expansion of pro-inflammatory chemokines may also be an evolutionary consequence of battles with pathogens, which would explain why constitutive chemokines are much less promiscuous because the ligands are not induced by the infectious agent. However, a recent study discovered that one of the ligands for CCR3, CCL11/Eotaxin, binds with high affinity to CXCR3 (66). This suggests that CXCR3 may act as a decoy receptor, sequestering locally produced CCL11/Eotaxin. In addition, the ligands for CXCR3, CXCL9/MIG, CXCL10/IP-10 and CXCL11/I-TAC have been found to inhibit CCR3-mediated functional responses. Consequently, chemokines that attract Th1 cells via CXCR3 can concurrently block the migration of Th2 cells in response to CCR3 ligands (67). These experiments demonstrate that some chemokines can bind receptors outside of their class, but appear to merely function antagonistically, or prevent the binding of other chemokines.

Chemokine Receptor	Main Ligand	Function	Sub-Class
CXC(α)			
CXCR1	CXCL8	Neutrophil Migration; Innate immunity; Acute Inflammation	ELR+
CXCR2	CXCL1,2,3,5,6,7,8	Neutrophil Migration; Innate immunity; Acute Inflammation; Angiogenesis	ELR+
CXCR3	CXCL9,10,11	Th1 Response, Angiostasis	ELR-
CXCR4	CXCL12	B Cell lymphopoiesis; bone marrow myelopoiesis; central nervous system and vascular development; HIV Infection	ELR-
CXCR5	CXCL13	B-Cell Trafficking; lymphoid development	ELR-
CXCR6	CXCL16	T Cell Migration	
CC(β)			
CCR1	CCL3,5,7,8,13,14,15,16,23	T cell and monocyte migration; innate and adaptive immunity; inflammation	
CCR2	CCL2,7,8,13	T cell and monocyte migration; innate and adaptive immunity; Th1 inflammation	
CCR3	CCL5,7,8,11,13,15,24,26	Eosinophil, basophil and T cell migration; allergic inflammation	
CCR4	CCL17,22	T cell and monocyte migration; allergic inflammation	
CCR5	CCL3,4,5,8,14	T cell and monocyte migration; innate and adaptive immunity; HIV infection	
CCR6	CCL20	Dendritic Cell Function	
CCR7	CCL19,21	Lymphocyte, Dendritic Cell Migration; lymphoid development; primary immune response	
CCR8	CCL1	T cell trafficking	
CCR9	CCL25	Lymphocyte Trafficking in Thymus and Small Intestine	
CCR10	CCL26,27,28	Lymphocyte Trafficking in Skin and Colon	
CX3C(δ)			
CX3CR1	CX3CL1	T and NK cell trafficking and adhesion; innate and adaptive immunity; Th1 inflammation	
C(γ)			
XCR1	XCL1,2	T cell trafficking	

Table 2: Chemokines and Chemokine Receptors

A list of CXC, CC, CX3C and XC chemokine receptors with their corresponding ligand(s) according to the systematic chemokine nomenclature system developed by Zlotnik and Yoshie in 1999. The function of each chemokine receptor pair and the ELR sub-class for CXC chemokines is also shown (10,20,68).

1.4 The Process of Leukocyte Extravasation

An inflammatory response initiates the trafficking of leukocytes from the micro-vasculature to the site of action in the tissue. Inflammatory stimuli initiate the response by activating the endothelial expression of selectins, integrin ligands and chemokines. Selectins are essential for the initial tethering of the leukocyte to the endothelium. An 'on' 'off' interaction between the selectins and their respective ligands provides a means for the leukocyte to 'roll'.

Firm adhesion is under the control of chemokines, lipid mediators and other pro-inflammatory molecules presented on the surface of the endothelium (Figure 3). Together, these molecules along with selectins activate adhesion molecules, namely the integrin ligands (ICAM-1, VCAM-1) which lead to firm adhesion.

Once firmly attached, the leukocytes transmigrate across the endothelium into the tissue, a complex process known as 'diapedesis'. In short, the leukocyte extends itself by a pseudopod across the endothelial border, which requires the disassembly and subsequent reassembly of its cytoskeleton. Platelet-endothelial-cell adhesion molecule-1 (PECAM-1), CD99, and the junctional adhesion molecule-A and C (JAM A/C), are present on both the leukocyte and endothelial junctions and provide homophilic (bind to same molecule) interactions that promote diapedesis (69). Diapedesis is also dependent on an increase in intracellular free calcium within the

endothelial cells between which the cells are passing (70,71). The rise in intracellular calcium has also been shown to activate myosin light chain kinases (MLCK) which are known to unfold myosin II and lead to endothelial cell retraction (72,73). There is also evidence that supports the notion that leukocytes can also migrate into sites of inflammation by passing directly through endothelial cells (74). Once the cells have extravasated, they may then be susceptible to chemokine gradients in tissues for directed cell migration to specific tissue locales, known as chemotaxis.

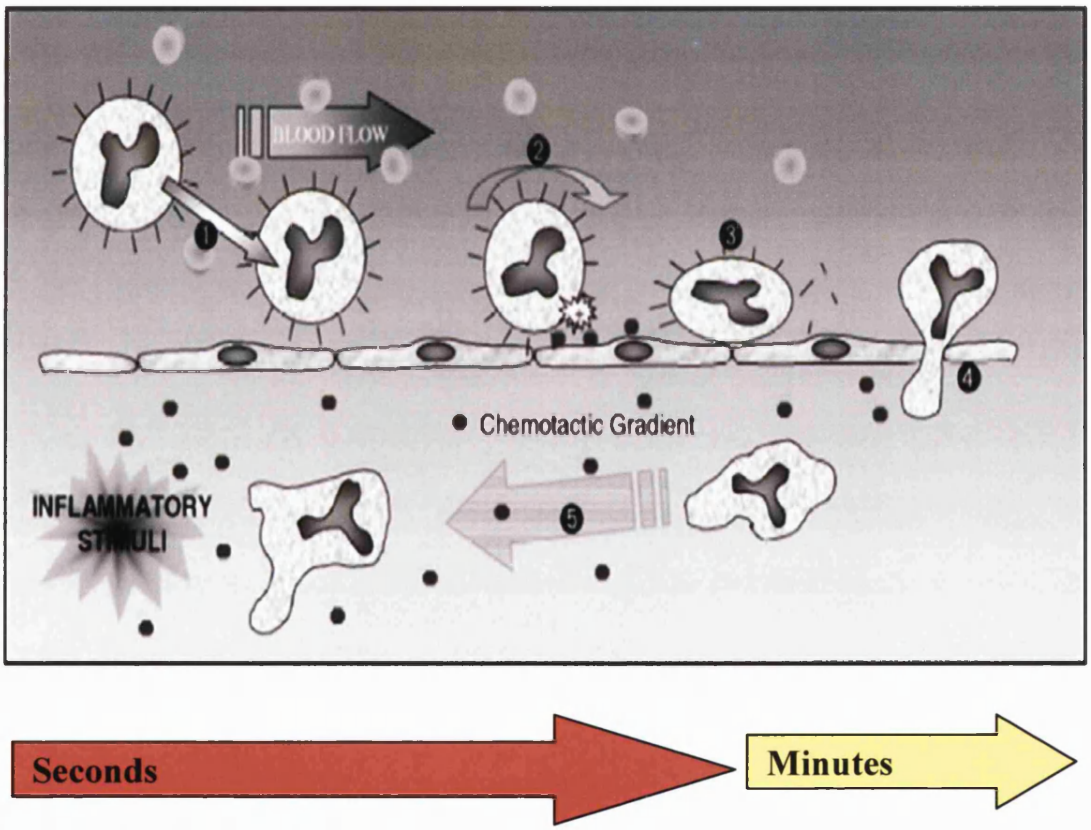

Figure 3. Leukocyte migration

The multi-step movement of leukocytes from the micro-vasculature to the tissue involves tethering (1), rolling (2), firm adhesion (3), diapedesis (4) and chemotaxis (5). All stages are under the tight regulation of inflammatory molecules (75).

Chemotaxis

Chemotaxis is the process by which cells detect the direction and intensity of, and move toward, an extracellular chemoattractant gradient. When cells sense a chemoattractant gradient, they dramatically change their shape, polarizing in the direction of the gradient (76). Polarity refers to the ability of a migrating cell to determine back from front. The leading edge protrudes at the cell front followed by the retraction of the rear edge (uropod) (77). This intriguing process plays a central role in development, immunity and tissue homeostasis (78-80). During embryogenesis, movements of cells in response to chemotactic stimuli bring form and organisation to tissues and organs and steer axons in the formation of the nervous system (81,82). In the immune system, an elaborate network of chemoattractants directs leukocytes to their correct locations and facilitates cell-cell interactions. Chemotaxis is also central to wound healing and has been implicated in disease states such as metastasis and atherosclerosis (83-85).

1.5 Tissue-specific Lymphocyte homing Signals

Tissue cells express specific chemokines on their surface to attract tissue-specific lymphocytes bearing corresponding chemokine receptors and adhesion molecules. It is evident that the circulating memory lymphocyte pool constitutes two clearly separable subsets of lymphocytes with skin versus gut homing potential. The skin-

homing circulating lymphocytes express cutaneous lymphocyte antigen (CLA), E-selectin, the integrin $\alpha_4\beta_1$, VCAM-1 (adhesion molecule) and the chemokine receptors CCR4 and CCR10. However, the gut homing lymphocytes express integrin $\alpha_4\beta_7$ and CCR9 (17).

More recently, a study investigating monocyte homing identified two principle subsets of blood monocytes that differ in their chemokine expression profile. The short-lived, inflammatory subset, express CCR2 and home to inflamed tissue to trigger immune responses. The resident subset with a longer half-life, express CX_3CR1 and home to non-inflamed tissues including liver, lung, brain and spleen. Here, the monocytes serve as precursors for resident myeloid cells, including $CD11c^+I\text{-}A^+$ dendritic cells (86).

Dendritic cells are known to switch chemokine receptor expression during maturation which permits their entry into secondary lymphoid organs whilst retaining immature cells in inflamed tissue. Immature dendritic cells express CCR1, CCR2, CCR5 and CXCR1 and migrate into inflamed peripheral tissues where they capture antigens. Maturing dendritic cells attain expression of CCR7, up-regulate CXCR4, down-regulate CXCR1 and lose expression of CCR1 and CCR5. Following maturation these cells migrate to the lymph nodes where they stimulate T cells (87).

So it is evident from the account above, that organ systems exhibit important differences in terms of their chemokine expression profile and are not immunologically uniform as first thought.

1.6 Genetically Modified Mice, Chemokines and Disease

The targeted deletion of chemokine receptors and the transgenic expression of their ligands have proven to be useful tools for determining the distinct biological role of these molecules *in vivo*. Table 3 and Table 4 outline the phenotypes that have been described for the various chemokines and chemokine receptors and Table 5 lists the effects seen following the transgenic expression of chemokines *in vivo*.

1.7 Differential expression of chemokine receptors within T cell subsets

The chemokine receptor expression profile differs within specific T cell subsets and will be discussed in more detail below.

Memory T Cells

In the last several years, functional diversity of the memory T-cell population has been attributed to memory T-cell subsets that differ in expression of homing and chemokine receptors. Two sets of human memory $CD45RO^+$ T cells have since been

been identified based on differential expression of the CCR7 chemokine and homing receptor. CCR7 expression on lymphocytes facilitates their migration into T cell areas of lymphoid organs in search of antigen presented by dendritic cells. Sallusto *et al.* designated the CD45RO/CCR7$^-$ subset as 'effector-memory' based on its ability to produce effector cytokines with rapid kinetics and the CD45RO/CCR7$^+$ subset as 'central memory' because of its predominant production of IL-2 and ability to differentiate into effector-memory T cells upon further stimulation (88). However, Unsoeld *et al.* (89) questions the proposed concept and argues that CCR7$^+$ and CCR7$^-$ memory T cells do not differ in their immediate effector cell function.

T Helper Cells

The existence of two functionally distinguished populations among T helper cells has also been established. Type 1 T helper (Th1) cells are involved in the defence against intracellular bacteria and many viruses, while type 2 Th cells (Th2) are the major actors in the response against parasites and play a central role in allergic inflammation. More recently, many studies have suggested that some chemokine receptors are tightly regulated on T cells, and in accordance with this selective expression, Th1 and Th2 cells can be differentially recruited by specific chemokines to the inflammatory sites. Th2-associated chemokine receptors include CCR3, CCR4 and CCR8, whereas Th1-associated chemokine receptors include CCR5 and CXCR3(90).

Chemokine Receptor	Phenotype of Knockout
CCR1 (91-96)	Imbalance in Th1/Th2 cytokines Reduced lung eosinophil recruitment in a murine model of asthma Resistance to EAE development
CCL3 /MIP-1α (97)	Mediates macrophage chemotaxis Enhances differentiation of primed CD8(+) T cells into effector cells and their release into circulation
CCR2 (98-105)	Reduced macrophage recruitment to inflammatory stimuli Increased resistance to atherosclerosis Decreased bronchial hyper-reactivity in a murine asthma model Resistance to EAE
CCL2/MCP-1 (106,107)	Imbalance of Th1/Th2 cytokines
CCR3 (108,109)	Eosinophils fail to traffick to intestinal mucosa but not lung Absence of eosinophils in a model of skin allergic inflammation
CCR4 (110-113)	Resistant to LPS-induced endotoxemia Attenuation of airway hyperresponsiveness during chronic pulmonary allergic responses to *Aspergillus*
CCR5 (114-118)	Partial resistance to LPS-induced endotoxemia Enhanced T cell-dependent immune responses Protecting and exacerbating effects on macrophage recruitment
CCR6 (119-121)	Reduced airway resistance, fewer eosinophils around airways, reduced IL-5 in lung and reduced serum IgE was observed in CA model Altered leukocyte homeostasis and cytokine environment in the intestinal mucosa 2-15-fold increase in specific T cell subsets within the mucosa
CCR7 (122,123)	Impaired B and T-lymphocyte migration Profound morphological alterations in all secondary lymphoid organs Upon activation DCs fail to migrate into draining lymph nodes
CCR8 (40-42)	Impaired Th2 cytokine production and eosinophil recruitment during Th2-mediated responses, with no abrogation of Th1-mediated responses No effect on the development of pulmonary eosinophilia and Th2 cytokine responses
CCR9 (124,125)	Intraepithelial T-cell-to-epithelial cell ratio is decreased in small intestine, effects of this are unknown Reduction in pre-pro-B cells but had no effect on generation of normal complement of mature B cells

Table 3. CC Chemokine and Chemokine Receptor Knock-Outs

This table summarises the main effects seen in CC chemokine and chemokine receptor knock-out mice. It should be noted that the phenotype of the CCR8 knockout mouse has been published by three independent groups however their findings contradict one another.

Chemokine Receptor	Phenotype of Knockout
CXCR2 (126-131)	Lymphadenopathy and splenomegaly, increased B cells Reduced neutrophilic infiltration in an acute model of gout Protection from septic injury Reduced progression of atherosclerosis
CXCR3 (132)	Profound resistance to development of acute allograft rejection
CXCR4 (133-135)	Lethal to embryo Critical in signalling progenitors to thymus for T cell differentiation Defective vascular development, haematopoiesis, cardiogenesis and derailed cerebellar neuronal migration.
CXCL12/SDF-1 (136,137)	Colonization of bone marrow by haematopoietic stem and myeloid cells severely impaired in embryo. Colonization of gonads by primordial germ cells also affected
CXCR5 (123,138)	Altered B-cell migration and germinal centre formation in spleen Lack of Peyer's patches
CX3CR1 (139,140)	Decreased atherosclerosis Selective reduction in natural killer cells

Table 4. CXC and CX_3C Chemokine and Chemokine Receptor Knock outs

This table summarises the main effects seen in CXC and CX_3C chemokine receptor knock-out mice. CXCR4 is the only receptor known to be embryonically lethal.

Chemokine	Effect of Transgenic Expression of Ligands
CXCL12/SDF-1α (137,141,142)	Enhanced marrow and splenic myelopoiesis Increased progenitor cell cycling and numbers Recruitment of dendritic cells (Promoter constructs used include: RSV and RIP7)
CCL3/MIP-1α (143)	Recruitment and activation of leukocyte populations (CMV promoter construct was used)
CXCL2/MIP-2 (144)	Inflammation of small intestine and colon (Fabpi promoter construct was used)
CCL2/MCP-1 (145-147)	Inflammatory Injury Recruitment of inflammatory infiltrate Transient and severe encephalopathy (Promoter constructs used include: MBP and GFAP)
CCL19/ELC (141)	Recruitment of lymphocytes and dendritic cells (RIP7 promoter construct was used)
CXCL10/IP-10 (148,149)	Recruitment of leukocytes to CNS Up-regulated in allergic pulmonary inflammation Contributes to airway hyperactivity and Th2 inflammation (GFAP) promoter construct was used)
CXCL13/BLC (141,150)	Lymphoid Neogenesis (RIP7 promoter construct was used)

Table 5. Transgenic Expression of Chemokine Ligands

This table summarises the main effects seen in response to the transgenic expression of chemokines.

1.8 Chemokine Signalling

Some of the underlying principles behind the differential expression of chemokine receptors have been discussed above. The question now is; how do chemokines and their receptors induce functional responses such as cell movement? The answer lies within the complex system of signalling pathways, which will be discussed below.

Signalling is a process employed by chemokine receptors to transduce information supplied by extracellular stimuli into intracellular second messengers that are deciphered as meaningful signals by the cell. It involves the coupling of agonist-activated G-protein coupled receptors (GPCRs) to a wide variety of effector systems via their interaction to heterotrimeric G proteins. The binding of agonist to a GPCR selects for a receptor conformation state that promotes the exchange of GDP for GTP on the G protein α-subunit and is presumed to allow the dissociation of the G protein G_{α}- and $G_{\beta\gamma}$- subunits (151).

The triggering of chemokines induces signalling pathways that may result in the control of a variety of biological functions including chemotaxis, proliferation, apoptosis and adhesion, to mention a few (12). These pathways are very complex and intertwined, and despite substantial advances in our understanding, they remain unclear. Many signalling pathways rely on the metabolism of plasma membrane

phosphoinositide lipids. The next section therefore describes phosphoinositide (PI) lipids and their role in signalling events.

1.9 Phosphoinositide Lipids

PI is the basic building block for the intracellular inositol-containing lipids, which consist of a D-myo-inositol-1-phosphate (Ins1P) linked via its phosphate group to diacylglycerol (Figure 4). The inositol head of PI has five free hydroxyl groups, however only those at positions 3, 4, and 5 can be phosphorylated, in different combinations. PI and its phosphorylated derivatives are collectively referred to as phosphoinositides or PI lipids. There are eight documented PI lipids, namely PI, mono-phosphates: PI(3)P, PI(4)P and PI(5)P, di-phosphates: $PI(3,4)P_2$, $PI(3,5)P_2$ and $PI(4,5)P_2$ and tri-phosphate: $PI(3,4,5)P_3$ (Figure 5). PI lipids all reside in membranes and are substrates for kinases, phosphatases, and lipases resident in or recruited to these membranes. One such enzyme that plays a key role in signal transduction is phospholipase C (PLC).

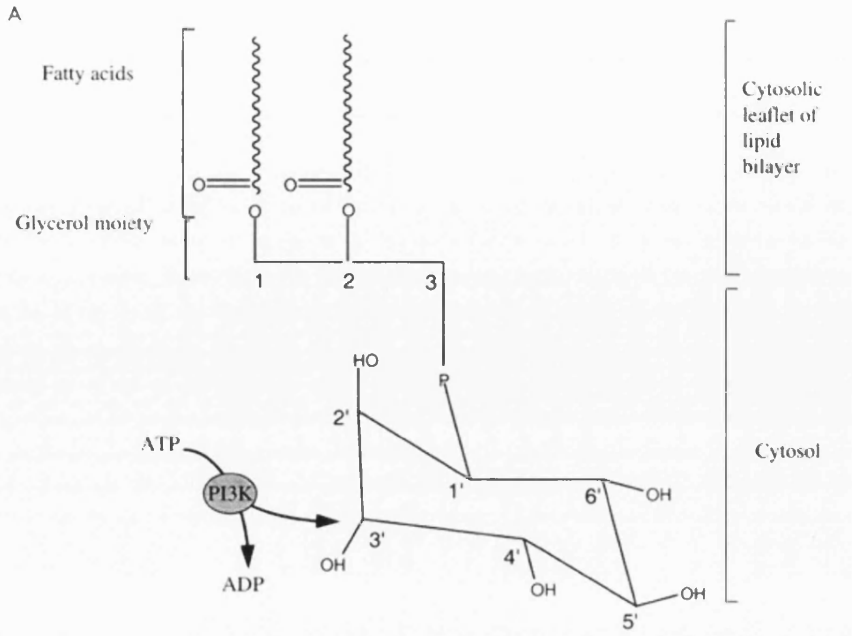

Figure 4. Chemical Structure of phosphoinositide (PI)

Phosphoinositide (PI) is the basic building block for the intracellular inositol-containing lipids, which consist of a D-myo-inositol-1-phosphate (Ins1P) linked via its phosphate group to diacylglycerol. The inositol head group of PI has five free hydroxyl groups, however only those at positions 3, 4, and 5 can be phosphorylated in different combinations (152).

1.10 Phospholipase C (PLC)

The PLC family is subdivided into PLCβ, -γ, -δ and -ε and differ in their structural organization and regulation. All members have multiple cellular regulators, but only the PLCβ enzymes are regulated by heterotrimeric G proteins. PLCβ signalling pathway is an important component in a network of signalling cascades that regulate cell function. Stimulation of PLCβ activity results in the rapid hydrolysis of $PI(4,5)P_2$, with production of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol

(DAG), intracellular mediators that increase intracellular calcium levels and activate protein kinase C (PKC) activity, respectively (153). Equally as important as PLC is the enzyme phosphoinositide 3-kinase (PI3K) which also catalyzes the formation of lipid second messengers.

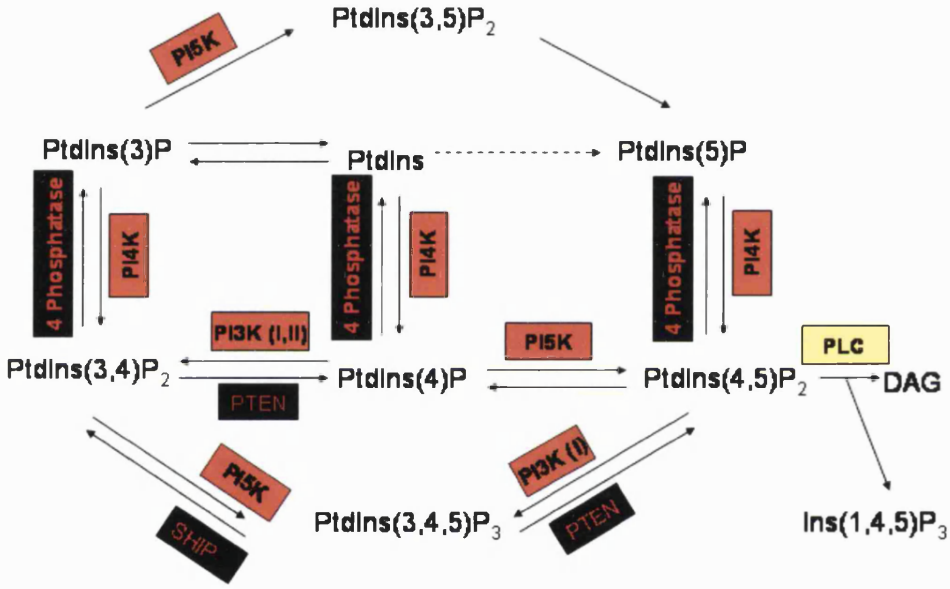

Figure 5. Phosphoinositide Lipid Metabolism.

The main pathways of PI synthesis and interconversions in mammalian cells. The black boxes with red text represent the phosphatases involved in the negative regulation of lipid metabolism. The red boxes with black text represent the enzymes that catalyse the phosphorylation of lipid substrates. (I) indicates the involvement of Class I PI3K and (II) indicates the involvement of Class II PI3K. PLC in yellow is an additional enzyme that cleaves $PI(4,5)P_2$ to produce diacyl glycerol (DAG) and $Ins(1,4,5)P_3$ (IP_3), important in the mobilization of calcium ions (154).

1.11 Phosphoinositide 3-kinase

Following activation by any number of extracellular stimuli (e.g. antigen, cytokines/chemokines, co-stimulatory molecules), PI3Ks catalyze the phosphorylation of PI lipids at the D3 position of the myo-inositol ring to produce phosphoinositide 3-phosphate (PI(3)P), phosphoinositide 3,4-bisphosphate ($PI(3,4)P_2$), and phosphoinositide 3,4,5-triphosphate ($PI(3,4,5)P_3$) (155) (Figure 5). These lipids selectively bind signalling molecules that contain specialised lipid binding motifs such as pleckstrin homology (PH), Phox homology (PX) and FYVE domains (Table 6). FYVE domains are generally specific for PI(3)P, whereas distinct PX domains, in the same way as PH domains, display distinctive specificities (156). Thus the production of $PI(3,4)P_2$ and $PI(3,4,5)P_3$ serve both to recruit signalling proteins to the plasma membrane and possibly induce (activating) conformational changes in proteins. The activity of these proteins can be regulated by the action of PI3Ks.

Classification of Phosphoinositide 3-kinase

The multiple isoforms of PI3Ks can be divided into three classes according to structure and substrate specificity. All PI3K catalytic subunits share a homologous region that consists of a catalytic core domain (HR1; homology region 1) linked to PIK domain (PI Kinase homology domain) and C2 domain (157) (Figure 6).

Selectivity	Domain	Protein
PI(3,4,5)P_3	PH	PKB
	PH	BTK
	PH	DAPP1
	PH	Grp1
PI(3,4)P_2	PH	PKB
	PH	DAPP1
	PH	TAPP-1
	PH	TAPP-2
PI(3)P	PX	Phagocyte NADPH oxidase (phox) complex
	FYVE	Fab1p
	FYVE	YOTB
	FYVE	Vac1p
	FYVE	EEA1

Table 6. Selectivity of PH Domain Containing Proteins.

This table describes the PH, PX and FYVE domain containing proteins that preferentially bind to PI(3,4,5)P_3, PI(3,4)P_2 and PI(3)P (158,159).

Class I PI3Ks

The class I group of PI3K is further divided into two sub-groups; prototypical class 1A and 1B. Class IA PI3K consists of a catalytic 110kDa subunit, which exists in a complex with an 85kDa adaptor/regulatory subunit. The 85kDa subunit is responsible for protein-protein interactions via the Src homology (SH2) domain, with phosphotyrosine residues of other proteins. The class IB family consists of a distinct lipid kinase termed PI3Kγ which is activated by G protein-coupled receptors but does

not interact with the SH2-containing adaptors that bind class IA PI3Ks. Instead the only identified member of this family, p110γ, associates with a unique p101 adaptor molecule (160). Class I PI3Ks are activated by a variety of extracellular stimuli, and have been implicated in a wide range of cellular processes, including cell cycle progression, growth, motility, adhesion and survival.

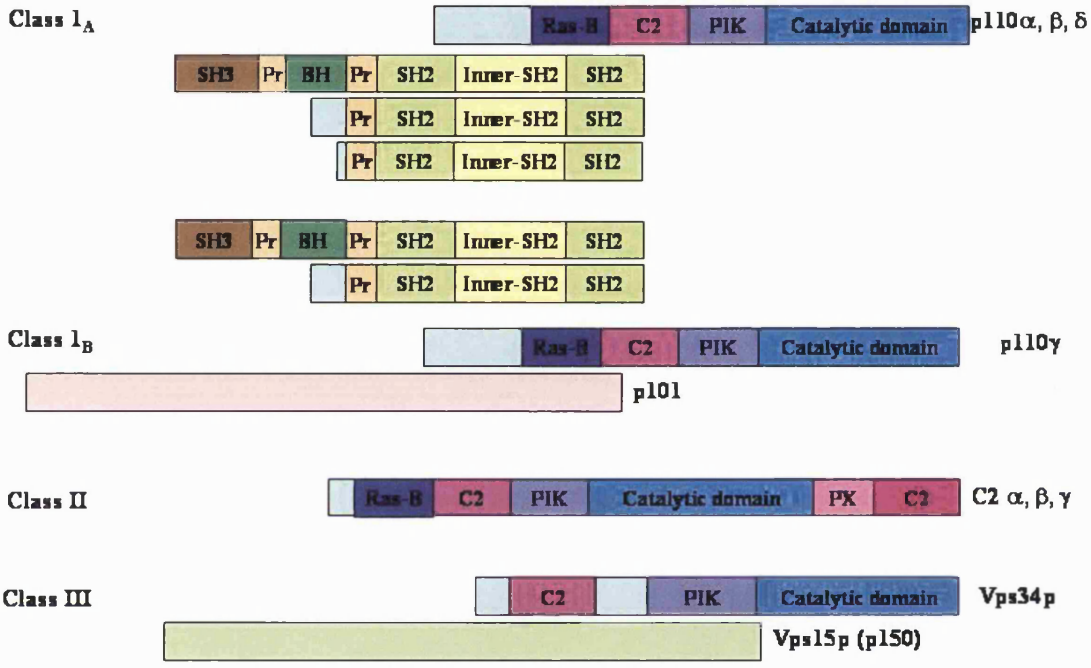

Figure 6 Structural Characteristics of the Class I, II and III PI3K Family Enzymes

The PIK domain is a helical domain that is found in lipid kinases but not protein kinases. C2 domains bind phospholipid in a calcium-dependent and -independent manner; those of PI3Ks do so in a calcium-independent fashion and may recruit PI3Ks to the membrane. The inter-SH2 domain of p85α p55α, p50α, p85β and p55γ constitutively interacts with the N-terminal domain of p110 α, β, γ. Dual SH2 domains bind to tyrosine-phosphorylated adaptor proteins, leading to activation of the kinase activity of the p110 subunits. p101 specifically interacts with the N-terminal domain of p110γ. PX domains are known to bind PI(3)P and may recruit the class II PI3K to the membrane. Functions of the Ras-binding domain (Ras-B), SH3 and Bcr domains and the proline-rich region (Pr) are not fully understood. Vps34p, the class III PI3K, forms a complex with a serine/threonine kinase, Vps15p (161).

The importance of PI3K in the signalling pathways that regulate these responses has been established through the use of pharmacological inhibitors such as wortmanin and LY294002, over expression of dominant-negative forms of PI3K or mutation of required sites on transmembrane receptors.

Class II PI3Ks

The class II PI3Ks (PI3K-C2α/β/γ) are approximately 170kDa, and their defining feature is a C2 domain at the carboxy terminus. Class II PI3Ks utilise predominantly PI and PI(4)P as substrates *in vitro*, but when PI lipid substrates are presented in combination with phosphatidylserine, they can also phosphorylate $PI(4,5)P_2$ (160).

Class III PI3Ks

The class III PI3K is the homologue of the vesicular-protein Vps34p found in yeast and of the p150 in mammals. Class III PI3Ks can utilise only PI as a substrate and are likely to be responsible for the generation of most of the PI3P in cells (152,157).

1.12 Protein Kinase B (PKB)

PKB is a serine/threonine kinase belonging to the 'AGC' superfamily of protein kinases (of which there are over 80 members). Interest in PKB has been highlighted by its role in promoting cell survival signals through the PI3K pathway, leading to inactivation of a series of pro-apoptotic proteins. In quiescent cells, PKB resides in the cytosol in a low-activity conformation. Upon activation of PI3K, $PI(3,4,5)P_3/PI(3,4)P_2$ are synthesised at the plasma membrane where PKB selectively interacts through its PH domain with these lipids (Figure 7) (162,163).

PKB is activated by, and dependent upon, multisite phosphorylation. Phosphorylation of PKB on Thr^{308} causes a charge-induced change in conformation allowing substrate binding and greatly elevated rate of catalysis. The phosphorylation of Thr^{308} strictly governs the activation of PKB, and its mutation to non-phosphorylatable alanine greatly reduces activity (164). There is convincing evidence that Thr^{308} is phosphorylated by the upstream activating kinase, 3-phosphoinositide-dependent kinase-1 (PDK1) (163,165). PDK1 phosphorylates PKB *in vitro*, and overexpression of PDK1 in cells also leads to elevated Thr^{308} phosphorylation in the absence of natural agonists.

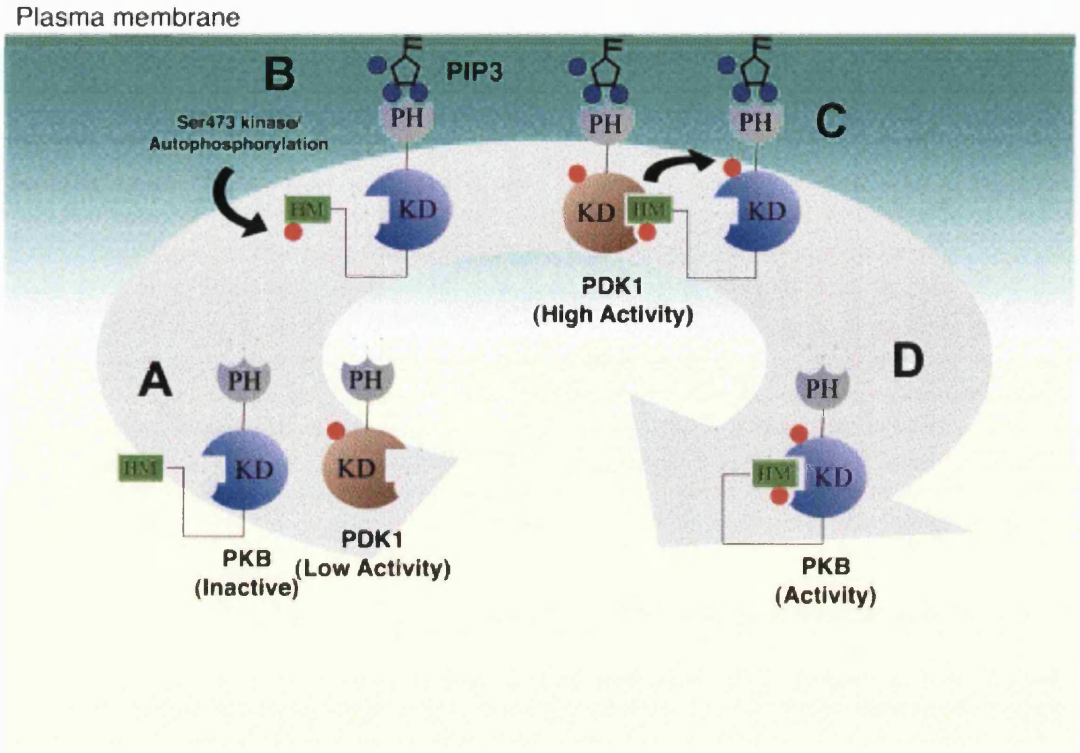

Figure 7. Activation Cycle of PKB

A: PKB is cytosolic and inactive. PDK1 has low activity, and Thr^{308} phosphorylation is not favourable. B: PI3K-generated $PI(3,4,5)P_3$ and $PI(3,4)P_2$ recruit PKB and PDK1 to the plasma membrane. PKB undergoes hydrophobic motif (HM) phosphorylation by the Ser^{473} kinase or by autophosphorylation. C: The HM of PKB stabilizes and activates PDK1, which then phosphorylates PKB on Thr^{308}. D: The HM of PKB associates with and stabilizes its kinase domain. The fully active kinase phosphorylates cytosolic and nuclear targets. HM, hydrophobic motif; PH, pleckstrin homology domain; KD, kinase domain. Red circles indicate phosphorylation events (166).

The second important regulatory domain of PKB is the C-terminal hydrophobic motif and its target residue Ser^{473} (167). The hydrophobic motif provides a docking site for PDK1, which if interrupted severely attenuates phosphorylation of the activation loop (168,169). More recent biochemical and structural studies have elegantly demonstrated that the hydrophobic motif also serves as an allosteric regulator of catalytic activity (170,171). Ser^{473} phosphorylation always parallels the activation of

PI3K and full activation of PKB however the mechanism by which this occurs is not completely understood. There is some evidence suggesting both PKB autophosphorylation (172) and phosphorylation by distinct serine kinases, including the integrin-linked kinase (ILK) (173).

Role of PKB in Cell Growth

PKB has been implicated in the maintenance of cell growth following a study in which its over-expression resulted in an anti-apoptotic effect and subsequent delay of cell death (174,175). This is evident in breast cancer where the expression of both PKBα and γ are up-regulated (176,177). To date, no satisfactory explanation has been provided as to how PKB delays cell death. Research has mainly focussed on finding direct links between PKB and the cell-death machinery.

Components of apoptotic machinery – targets of PKB

The following paragraphs will summarise the studies that have discovered links between PKB and cell death.

BAD (Bcl-2/Bcl-X_L-antagonist, causing cell death)

BAD forms a heterodimer with the anti-apoptotic proteins Bcl-2 or Bcl-X_L preventing them from exerting their anti-apoptotic function. PKB is known to phosphorylate BAD which leads to the dissociation of the heterodimer and enables Bcl-2 or Bcl-X_L to block apoptosis (178).

Forkhead (FH) transcription factors

The link between PKB and forkhead (FH) transcription factors (FKHR, FKHRL1 and AFX) was first established in *C. elegans*, where the insulin receptor/PI3K/PDK1/PKB pathway was shown to suppress the action of the DAF16 gene which encodes a transcription factor belonging to the FH family. Phosphorylation of FH transcription factors by PKB promotes their translocation from the nucleus into the cytoplasm where they interact with 14-3-3 proteins, effectively holding them from their target genes in the nucleus (179).

IκB Kinases (IKKs)

NF-κB is sequestered in the cytoplasm when bound to the cytosolic inhibitor IκB. PKB has been reported to associate with and activate IκB kinases (IKKs) which phosphorylate and induce the degradation of IκB. This permits the translocation of

NF-κB to the nucleus where it activates the transcription of anti-apoptotic proteins such as inhibitor-of-apoptosis (IAP) proteins c-IAP1 and c-IAP2 (180).

Possible link between PKB and Raf protein kinase

The mitogen-activated protein-kinase (MAPK) pathway regulates many physiological processes such as proliferation, differentiation and apoptosis (discussed in detail in 1.16). This pathway is highly reliant on the translocation and activation of Raf to the plasma membrane by its interaction with activated Ras. Recent studies indicate that PKB can inhibit Raf by phosphorylating it at Ser^{259}. This leads to the interaction of Raf with 14-3-3 proteins resulting in an inhibition of the Raf-MAPK signal transduction pathway (181). However, in a significant number of cell lines, PI3K inhibitors which block PKB, either have no effect on agonist-induced Raf activation or in some cases actually inhibit the activation of Raf, suggesting that PKB can contribute to Raf activation under certain circumstances (182,183).

1.13 PI3K dependent signalling

Numerous studies using PI3K inhibitors, overexpression of mutated forms of PI3K and, more recently, gene knockout experiments in mice, have implicated PI3Ks in the regulation of a diverse array of cellular responses, including cell survival,

mitogenesis, membrane trafficking, glucose transport, membrane ruffling and superoxide production, as well as actin reorganization and chemotaxis (157). Elegant molecular and pharmacological evidence first suggested that PI3K and its lipid products might play an important role in platelet-derived growth factor (PDGF)-dependent actin polymerization and cell migration (184-187). Moreover, selective activation of PI3K using constitutively active PI3K mutants or the addition of exogenous $PI(3,4,5)P_3$ can initiate cell motililty and membrane ruffling (188,189).

Chemokine-mediated Lipid Accumulation

The technique commonly used to assess the activation of PI3K, is to measure the accumulation of its lipid products following receptor stimulation. The method relies on metabolic labelling of intact cellular pools of ATP with [^{32}P]-Orthophosphate, receptor stimulation, followed by lipid extraction (190,191) and separation of the [^{32}P]-labelled-D3 PI lipids by high-performance liquid chromatography (HPLC) analysis (192). Utilising this method, CXCL12/SDF-1 and certain CXCL12/SDF-1 peptide analogues have been shown to stimulate the transient accumulation of $PI(3,4,5)P_3$ in leukaemic T-cell lines and peripheral blood-derived T lymphocytes. In both cell types, the elevation of $PI(3,4,5)P_3$ was found to be rapid and transient, being detectable within 15 seconds post-stimulation and returning to basal levels within 5 min after chemokine treatment. Other studies have investigated the effect of CCL2/MCP-1 stimulation on PI lipid accumulation in the monocytic cell line THP-1.

This model revealed that CCL2/MCP-1 was also able to elicit rapid and transient accumulation of $PI(3,4,5)P_3$. It appears therefore that at least two different chemokines which bind to distinct receptors are able to activate PI3K in different cell systems.

The very first evidence for the involvement of PI3K in chemokine-stimulated cell migration was the demonstration that chemotaxis and polarization of T cells induced by CCL5/RANTES could be inhibited by PI3K inhibitors such as wortmannin and LY294002 (193). Subsequent studies by several groups have shown that other CC chemokines (e.g. CCL20/MIP-3α and CCL2/MCP-1) as well as CXC chemokines (e.g. CXCL1/IL-8 and CXCL12/SDF-1) stimulate wortmannin-sensitive chemotaxis of eosinophils, THP-1 cells, as well as neutrophils and T lymphocytes, respectively (194-197). Thus, it seems probable that the production and degradation of 3-PI lipids is crucial in maintaining chemotactic signalling gradients.

The role of PI3K isoforms in chemokine-mediated $PI(3,4,5)P_3$ Accumulation

It is a common phenomenon that chemokine receptors are G-protein coupled, which implicates PI3Kγ in mediating $PI(3,4,5)P_3$ accumulation. CXCL12/SDF-1 and CCL2/MCP-1-mediated accumulation of $PI(3,4,5)P_3$ were found to be abrogated following pre-treatment with the $G_{\alpha i}$ specific inhibitor, pertussis toxin (PTX)

(198,199). In addition CXCL12/SDF-1 and CCL2/MCP-1 have also been shown to stimulate an increase in the *in-vitro* lipid kinase activity present in anti-PI3Kγ immunoprecipitates derived from Jurkat and natural killer cells respectively (195,200). Leukocytes derived from genetically engineered mice deficient in PI3Kγ also fail to produce $PI(3,4,5)P_3$ in response to CXCL1/GROα (201). However, this study did not investigate the effects of CXCL12/SDF-1-mediated $PI(3,4,5)P_3$ accumulation in these knock-out mice. So it seems that PI3Kγ plays an important role in chemokine-mediated accumulation of lipids, however it does not appear to be the only isoform to be activated by chemokines. The p85/p110 heterodimer has been shown to be activated by CXCL12/SDF-1 and CCL5/RANTES in T cells (193,195) and by CCL2/MCP-1 in THP-1 cells (197). In addition, class II PI3K has been implicated as a down-stream effector of chemokine receptors, as demonstrated by the activation of PI3K-C2α in response to CCL2/MCP-1 stimulated THP-1 cells (197).

1.14 Regulation of PI3K-dependent pathways

PI3K-mediated signalling, in addition to its positive regulation, is subject to potent negative regulation by two inositol lipid phosphatases responsible for the degradation of $PI(3,4,5)P_3$; namely the 5' phosphatase SHIP (Src homology 2 domain-containing inositol polyphosphate) and the 3' phosphatase PTEN (Phosphatase and tensin homologue deleted on chromosome 10) (167,202,203).

SHIP 1/2

SHIP1 is a haematopoietic-restricted enzyme that plays a crucial role in the negative regulation of immunocompetent cells (204). A study using leukaemic cells demonstrated that a mutation within the active site of SHIP1 resulted in an enhanced PKB phosphorylation, promotion of cell survival and resistance to apoptosis (205). SHIP2, whose expression is ubiquitous, has been implicated in the genetic susceptibility of type 2 diabetes (206-208).

PTEN

PTEN plays a pivotal role in the down-regulation of PDK-1 and PKB-dependent pathways. Mutations that abolish its catalytic activity therefore result in increased cell survival, growth and proliferation (209-211). Failure to initiate inhibitory feedback mechanisms results in profound immune defects ranging from hyper-proliferation (as seen in cancers), to debilitating autoimmunity (212).

Regulation of PI3K within a B Cell Model

The regulation of PI3K-dependent signalling in B cells is a well characterized model system, based on two important receptors that are expressed on B cells, namely the B cell receptor (BCR) and the low affinity receptor for the Fc portion of immunoglobulin G (IgG), FcγRIIb. The FcγRIIb receptor enables B cells to

discriminate between free antigen, and immune complexes composed of antigen bound to IgG antibodies and thus dampen active humoral immune responses (213). Immune complexes co-ligate the BCR with the FcγRIIb receptor inducing src kinase, lyn-dependent phosphorylation of the immunoreceptor tyrosine-based inhibitory motif (ITIM), located in the cytoplasmic domain of FcγRIIB (213-215). Subsequent recruitment of active SHIP to the membrane results in the enzymatic degradation of PIP_3, preventing translocation of PH domain containing proteins such as Btk (216-221)(Figure 8).

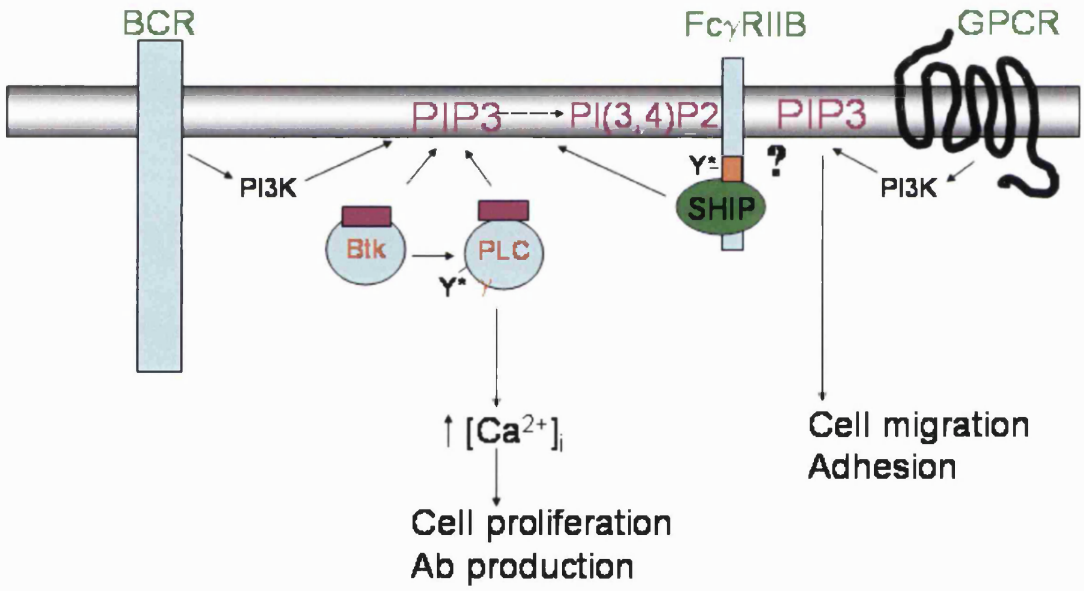

Figure 8. Model for the Negative Regulation of PI3K Signalling in B Cells

Activation of the BCR mediates a PI3K-dependent accumulation of $PI(3,4,5)P_3$ (PIP3). This induces the translocation of PH domain containing proteins such as Btk to the membrane leading to the activation of calcium-dependent responses. Co-ligation of the BCR and FcγRIIb receptor, in short, leads to the recruitment of SHIP which de-phosphorylates PIP_3 to $PI(3,4)P_2$, thus attenuating the responses downstream of the BCR.

Regulation of the MAP Kinase Pathway

FcγRIIB-mediated inhibition of the MAP kinase ERK1/2 may occur as a result of the catalytic or adaptor function of SHIP. Firstly, inhibition of ERK1/2 may be attributable to the SHIP-mediated reduction in $PI(3,4,5)P_3$ accummulation, by inhibiting the recruitment of Btk to the membrane. Btk-dependent PLCγ activation cleaves $PI(4,5)P_2$ generating the second messengers IP_3 and DAG, required for calcium mobilization and PKC activation respectively (222,223). The subsequent decrease in PKC activation contributes to the inhibition of ERK1/2 as found by Hashimoto *et al.* (1998) (224).

Secondly, the adaptor function of SHIP exposes binding sites for the SH2-domain containing Shc or the phosphotyrosine binding (PTB) domains of $p62^{dok}$ (Figure 9). Following a BCR induced signal, Shc forms a complex with Grb2 and Sos that leads to the activation of the Ras-mediated ERK1/2 MAPK pathway. However, an inhibitory response promotes the association of Shc with SHIP preventing the formation of this complex and thus inhibiting the phosphorylation of ERK1/2 (225). Alternatively the scaffolding effect of active SHIP, $p62^{dok}$ and RasGAP leads to the inhibition of Ras-mediated ERK1/2 by reducing Ras_{GTP} to Ras_{GDP} (226,227). Ultimately, SHIP affects proliferation, cell survival, protein synthesis and multiple other effector functions (228).

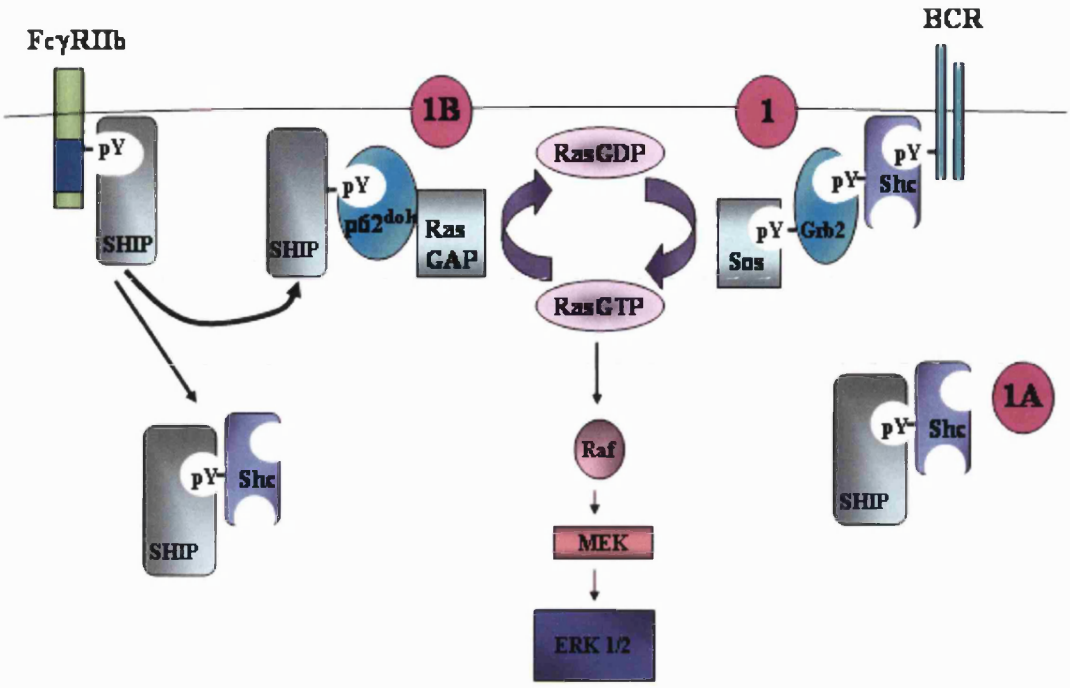

Figure 9. A Diagram High-lighting the Adaptor Function of SHIP in the Inhibitory Regulation of BCR-mediated ERK1/2 Phosphorylation

Stimulation of the BCR with antigen leads to activation of the ERK1/2/MAPK pathway via assembly of the Shc/Grb2/Sos complex (1). Two mechanisms involving the adaptor function of SHIP have been proposed to inhibit the ERK1/2/MAPK pathway: SHIP competes with Grb2 for binding to Shc and blocks the formation of the Shc/Grb2/Sos complex (1A); or SHIP is recruited to the FcγRIIb receptor and binds the RasGAP docking protein $p62^{dok}$, inhibiting the ERK1/2/MAPK pathway through inactivation of Ras (1B).

It is evident from the account above that SHIP is imperative in the inhibitory regulation of BCR-mediated responses; however it is unclear as to whether SHIP may regulate responses on a more global basis. Given that signalling through CXCR4 is known to require $PI(3,4,5)P_3$ in both T (229) and B cells (230), it is plausible that FcγRIIb-mediated recruitment of SHIP may regulate chemokine-induced responses.

1.15 Calcium

As described above, PI3Ks play an important role in the regulation of many signalling pathways that are reliant on binding to D3-phosphorylated phosphoinositides. However, PI3Ks are not the only molecules that participate in the positive regulation of responses. Calcium acts as an intracellular second messenger, relaying information within cells to regulate their activity. The versatility of calcium is reflected in the diverse functions with which it is involved, including fertilization, cell development and differentiation, mediation of cell activity and death. The following section describes the role, function, regulation and release mechanisms of calcium from internal stores.

Calcium is a ubiquitous second messenger involved in the regulation of a variety of cellular processes, including gene transcription, proliferation and muscle contraction. The mechanisms that regulate the intracellular Ca^{2+} concentration include channels located at the endoplasmic (ER) and sarcoplasmic reticulum (SR), as well as at the plasma membrane which regulate the supply of Ca^{2+} from the stores and extracellular space respectively. Removal of calcium is achieved by the combined action of buffers (calmodulin, calbindin and parvalbumin), pumps (Ca^{2+}-ATPase) and exchangers (Na^{+}/Ca^{2+} on mitochondria) (231).

Calcium Release from Internal Stores

The two major intracellular stores of calcium are primarily found in the endoplasmic reticulum (ER), which specialize in the accumulation, storage and release of calcium (Figure 10). Channels which control the release of calcium from the stores include the IP_3 (IP_3R) and ryanodine receptors (232,233). IP_3, known to activate the IP_3R, is cleaved along with diacyl glycerol (DAG) from $PI(4,5)P_2$ by the enzyme PLC (231). The isoform of PLC involved is dependent on the activation mechanism. For example, G-protein-coupled receptors are known to activate PLCβ, tyrosine-kinase-coupled receptors activate PLCγ, a rise in calcium concentration activates PLCδ and stimulation through Ras activates PLCε (234). Pharmacological inhibitors such as the PLC inhibitor, U-73122 (IC_{50} between 1.0 - 2.1 μM), its less effective analogue U-73343 and the IP_3 inhibitor, 2APB can be used to determine whether the intracellular calcium release in response to receptor stimulation occurs in this way. The Ca^{2+} activating ryanodine receptor, modulated by cyclic ADP ribose (cADPR) (235) is also activated by low concentrations of ryanodine, however, at high concentrations, ryanodine has been shown to inhibit the release of calcium from the stores (236).

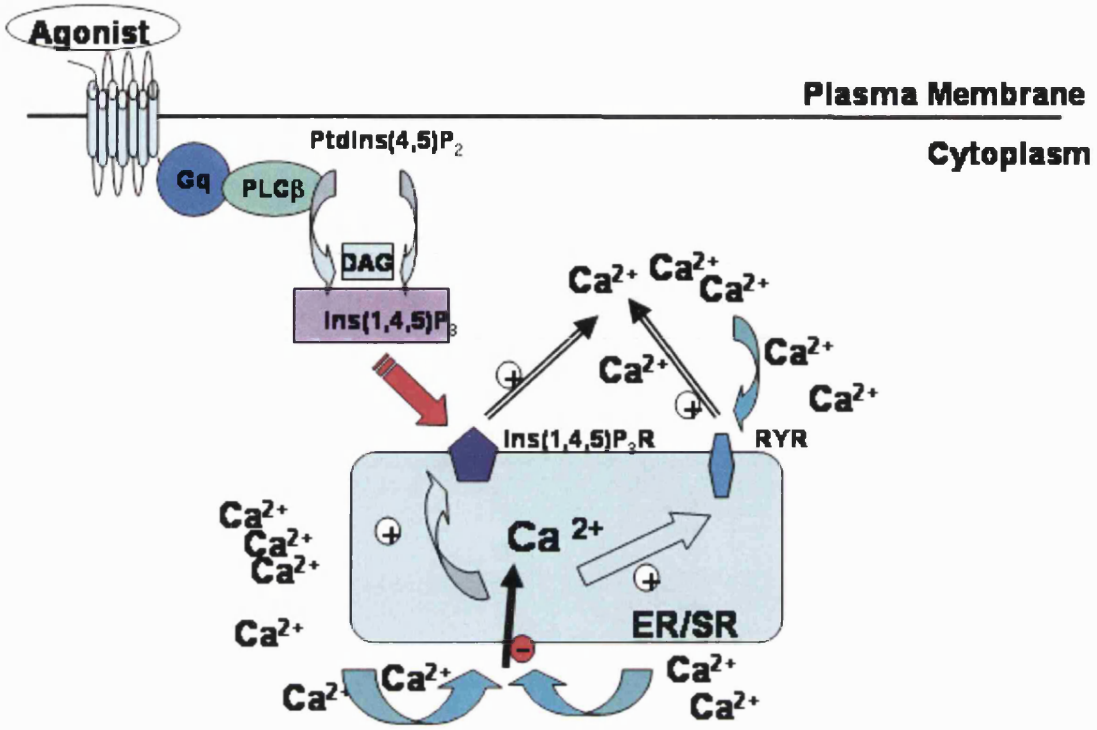

Figure 10. Calcium Release from Internal Stores

Various second messengers or modulators regulate the release of calcium from internal stores by the inositol-1,4,5-trisphosphate receptor ($Ins(1,4,5)P_3R$) or the ryanodine receptor (RYR). These release channels are sensitive to factors that function from the cytosol and from within the lumen of the endoplasmic/sarcoplasmic reticulum (ER/SR). The $Ins(1,4,5)P_3R$ is regulated by inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$, which is generated by various signalling pathways by PLCβ.

1.16 MAP-kinase Pathway

One of the most studied signalling pathways described so far is the MAPK pathways, which are known to control intricate programs, such as embryogenesis, differentiation, proliferation, homeostasis and cell death (237,238). The MAP-kinases are a family of serine/threonine kinases used by eukaryotic cells to transmit

extracellular signals from the membrane to nuclear and other intracellular targets thereby regulating gene expression (239,240). In mammalian systems five distinguishable MAPK modules have been described so far (Figure 11). These include the extracellular signal-regulated kinase ERK1/2 (p42/44) (241) which preferentially regulate cell growth and differentiation, as well as the c-Jun NH_2-terminal kinase (JNK p46/54) (242) and p38αβγδ (243) which function mainly in stress responses like inflammation and apoptosis, and regulate a large variety of cellular processes such as cell growth, differentiation, development, cell cycle, death and survival (244). The pathways differ in their upstream activation sequence and in their downstream substrate specificity but are all organized in a three-kinase architecture consisting of a MAPKK-kinase that phosphorylates and activates a MAPK-kinase, which in turn activates MAPK (Figure 11). The arrangement of this pathway promotes signal amplification.

Multiple messengers acting through GPCRs lead to the activation of MAPK cascades, including ERK1/2 (245). The role of G-protein subunits in the activation of this pathway will now be discussed.

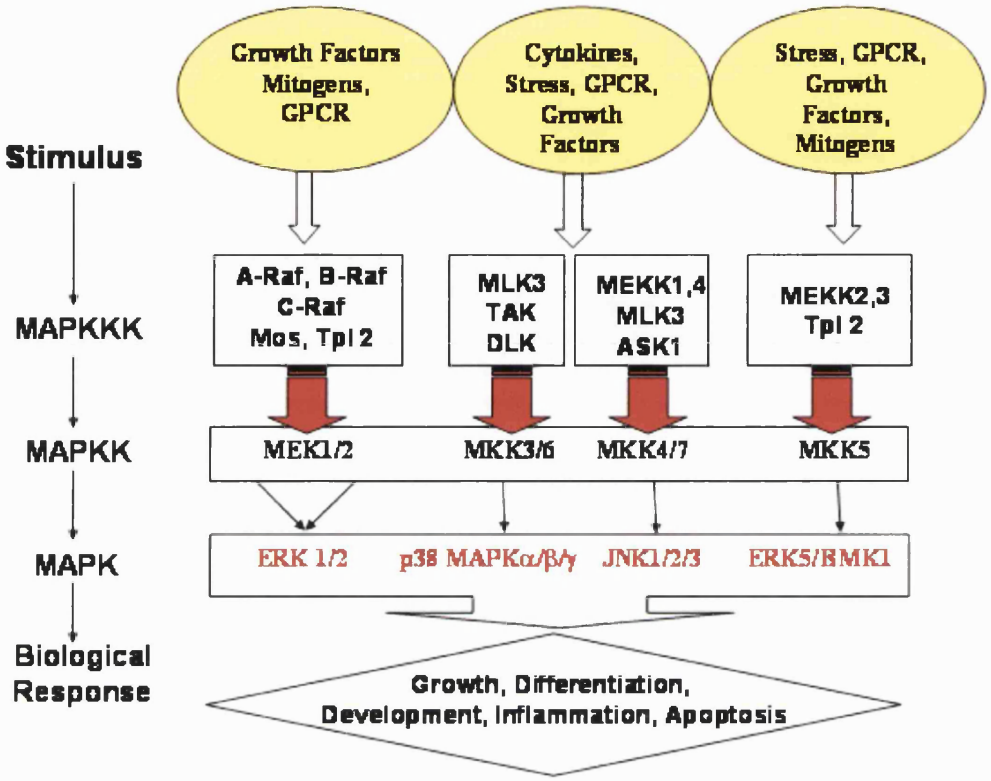

Figure 11. Mammalian MAP-kinase Pathway

The MAP-kinase pathways differ in their upstream activation sequence and in their downstream substrate specificity but are all organized in a three-kinase architecture consisting of a MAPKK-kinase that phosphorylates and activates a MAPK-kinase, which in turn activates MAP-kinase, that ultimately leads to activation of transcription factors.

The Link between GPCRs and MAPK Pathways

GPCRs are known to interact with heterotrimeric G proteins, that include α-, β-, and γ subunits. Receptor-mediated exchange of GTP on the α-subunit of the heterotrimer causes the 'activated' α-subunit to dissociate from the 'free' β-γ- subcomplex (151).

Both of these components are active signalling molecules that modulate an array of downstream effectors. The G_α proteins are classified into four different families – $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$ and $G_{\alpha 12}$.

The primary effect of the $G_{\alpha s}$ family is to stimulate the intracellular messenger adenylyl cyclase (AC), which catalyzes the conversion of cytoplasmic ATP to cyclic AMP (cAMP). cAMP has been claimed to activate the small GTP-binding protein Rap1 which links to the MAPK pathway through the activation of B-Raf (246). $G_{\alpha i}$ and $G_{\alpha q}$-coupled receptors can stimulate the MAPK in either a PKC-dependent and Ras-independent manner or in a PKC-independent but Ras-dependent manner. Second messengers generated as a consequence of $G_{\alpha i}$ and $G_{\alpha q}$ stimulation can provoke Ras activation through RasGRF and RasGRP, two tissue-restricted guanine exchange factors (GEFs) for Ras and Ras-related GTPases (Figure 12).

$G_{\beta\gamma}$ subunits have been shown to induce ligand-independent tyrosine phosphorylation of receptor tyrosine kinases (RTKs) such as EGFR (247,248) and PDGFR (249). This, known as 'Transactivation', is thought to occur either in a PI3Kγ-dependent or independent manner (250) via the non-receptor-tyrosine-kinase (NRTK) Src (251), resulting in the formation of the Shc-Grb2-Sos complex. Other NRTKs, which include C-terminal Src kinase (Csk), Lyn, Bruton's tyrosine kinase (Btk) and proline-rich tyrosine kinase 2 (PYK2), have also been proposed to mediate the activation of MAPK by $G_{\alpha i}$- and $G_{\alpha q}$-coupled receptors in a variety of cell types (246) (Figure 12).

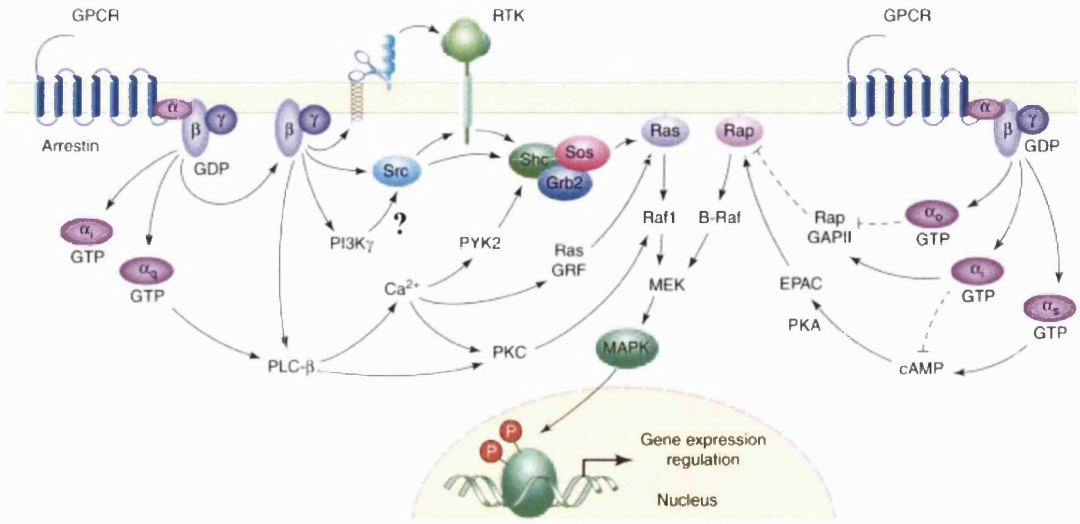

Figure 12. Multiple pathways link GPCRs to MAPKs

Biochemical routes initiated by βγ-subunits can stimulate Ras by the activation of receptor and non-receptor tyrosine kinases, which results in the recruitment of Sos to the membrane and the exchange of GDP for GTP bound to Ras. Activated $G\alpha_q$ can stimulate Raf1 through protein kinase C (PKC) or by stimulating Ras by the Ca^{2+}-dependent activation of RasGRF and tyrosine kinases acting on Sos. $G\alpha_i$, $G\alpha_o$ and $G\alpha_s$ can also use tissue-restricted pathways regulating Rap, which can stimulate B-Raf and lead to the activation of MAPK. Activated MAPK translocates to the nucleus and phosphorylates nuclear proteins, including transcription factors, thereby regulating gene expression. Arrows represent positive stimulation and broken lines represent inhibition. Abbreviations: EPAC, exchange protein activated by cAMP; GAP, GTPase-activating protein; GRF, guanine-nucleotide releasing factor; MEK, MAPK kinase; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PLC, phospholipase C; RTK, receptor tyrosine kinase (252).

1.17 CXCL12 mediated Signalling through CXCR4

CXCR4 is one of the best described members of the large family of 7-transmembrane domain receptors coupled to heterotrimeric G_i proteins (253,254). The exclusive

ligand for CXCR4, CXCL12/SDF-1; was first described as a powerful chemoattractant for peripheral blood lymphocytes (255), $CD34^+$ progenitor cells (256) and pre and pro-B cell lines (257). CXCL12-mediated CXCR4 leads to the activation of a multitude of signalling molecules which result in the establishment of diverse signalling pathways. Increased phosphorylation has been observed in the focal adhesion molecules, Pyk-2, p130Cas, focal adhesion kinase (FAK), paxillin, Crk and Crk-L (258). In addition CXCL12 has been shown to induce the activation of ERK1/2, PLCγ, PKC, PI3K, Janus kinase signal transducers, and NFκB (258). CXCL12 triggers the C-terminal-mediated internalization of CXCR4, involving G protein-coupled receptor kinases (GRKs), followed by binding of β-arrestin (254,259). This process is not only essential for the desensitisation of membrane bound receptors but is also thought to activate several pathways and functions, such as chemotaxis (260) and ERK1/2 (261,262). There are however conflicting studies which indicate that chemotaxis (263) and ERK1/2 activation (264) are independent of GPCR endocytosis.

1.18 CCL1 mediated Signalling through CCR8

CCR8-dependent signal transduction pathways are ill defined; to date only activation of the Ras/MAPK pathway is shown to mediate anti-apoptotic activity of CCL1 and vMIP-I (265). We set out to further define the pathways responsible for the phosphorylation of ERK1/2 and PKB, and determine possible routes in which CCL1

mediates calcium mobilization and chemotaxis in either CCR8-transfected Rat Basophilic Leukaemia cells or in the T cell line HUT-78.

Signalling pathways are not only regulated by lipid phosphatases but also by the recycling of receptors. This process involves receptor internalisation, re-sensitization, recycling and degradation, all of which will be discussed in more detail below.

1.19 Receptor Desensitization

Ligand binding to GPCRs not only results in their activation but also in their desensitization. Desensitization is an adaptive response used by cells to arrest G protein signalling, therefore preventing the potentially harmful effects that can result from persistent receptor stimulation. The universal mechanism employed by all cells, involves the coordinated actions of two families of proteins, the G protein-coupled receptor kinases (GRKs) and the arrestins (266) (Figure 13). GRKs phosphorylate the serine and threonine residues of agonist-occupied GPCRs in their third intracellular loop and C-terminal domains. Since GRK phosphorylation alone has little effect on receptor-G-protein coupling in the absence of arrestins, the role of GRK phosphorylation appears to increase the affinity of the receptor for arrestins (267). Based upon mutagenesis studies, the β-arrestins are comprised of two major functional domains, an N-terminal domain responsible for recognition of activated GPCRs and a C-terminal domain responsible for secondary receptor recognition

(268). β-arrestin binding sterically precludes coupling between the receptor and heterotrimeric G proteins, leading to termination of signalling by G proteins effectors (269). Receptor-bound β-arrestins also act as adapter proteins, binding to components of the clathrin endocytic machinery including clathrin and β2-adaptin (AP-2). (270,271). Formation of the clathrin coated vesicle (CCV) is dependent on the cytoplasmic GTPase dynamin as discovered using dominant-negative mutants of dynamin which blocked the internalization of EGF receptors (272). Once internalised the receptor complex is transported by the clathrin-coated vesicle to early endosomes, a diverse population of membrane compartments with tubular-vesicular morphology (273). The endosomes sort receptor-complexes and deliver them to either late endosomes for divergent recycling or lysosomes for degradation. In general, receptor endocytosis results in the rapid desensitisation of signalling activity. This can either be restored by receptor recycling which promotes the re-sensitisation of receptors, or prolonged following lysosomal degradation (274). The key factors, which dictate the outcome of the receptor, include the de-phosphorylation of residues on the cytoplasmic tail of the receptor which promotes recycling (275) and ubiquitination. Ubiquitination is a process in which ubiquitin modifies lysine residues located in the carboxyl-terminal domain and promotes the trafficking of endocytosed receptors to the lysosomes (276).

Regulation of Endosomes by Rab GTPases

Intracellular trafficking of membrane receptors is tightly regulated by a subfamily of Rab GTPases. The family of Rab GTPases consist of 60 members and are known to regulate vesicle formation, motility, docking and fusion within the endocytic and secretory pathways (277). Among them, Rab 5 is considered to transport and catalyze the fusion of endocytic vesicles with the early endosome. Rab 4 is localised in the early and recycling endosomes, where it ensures the recycling tubules return and fuse to the early endosome compartment in a Rab 5 dependent manner. Rab 11 however, is primarily found in association with the recycling endosomes and the trans-Golgi network (TGN) and regulates trafficking through these compartments (278). Similarly, Rab 7 and 9 occupy distinct domains within late endosomes (279).

Internalisation of CXCR4

CXCR4 has the capacity to interact with the endocytic adaptor β-arrestin (259). β-arrestin interacts with the C-terminus of CXCR4 and results in the attenuation of chemokine-mediated G protein activation.

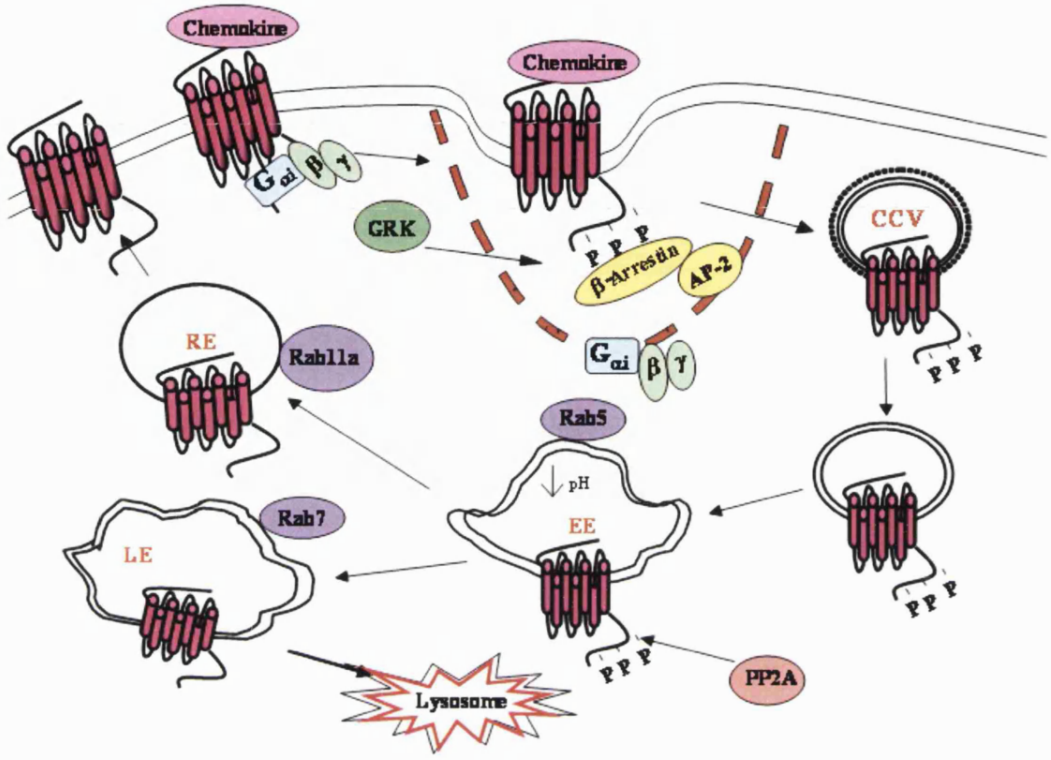

Figure 13. Receptor Mediated Endocytosis.

The mechanism of receptor cycling is a multi-step process regulated by adaptors such as β-arrestin and AP2. β-arrestins sterically prevent receptor interaction with heterotrimeric G proteins and directly link receptors to clathrin-coated pits. The clathrin coated pits are small areas of the plasma membrane that can invaginate inwards, and recruit ligand (chemokine) bound receptor complexes for internalisation. Clathrin-coated vesicles (CCV) are formed and enter the cytoplasm where they become un-coated (clathrin removed) and fuse with specialised membrane organelles known as endosomes (Early Endosome EE, Late Endosome LE and Recycling Endosome RE). The endosomes are responsible for the sorting of receptors, which are either removed from the cell by lysosomes or recycled back to the plasma membrane. These compartments are under the tight control of Rab GTPases (Rab 5, 7 and 11a).

The role of Class I PI3K in GPCR internalisation

In addition to phosphorylating activated β adrenergic receptors (βARs) the GRK β adrenergic receptor kinase 1 (βARK1) recruits class I PI3Ks to the receptor complex. This leads to an increase in the production of D3 lipid molecules which regulate the recruitment of the clathrin adaptor adaptin-2 (AP-2), and the receptor/β-arrestin complex, to clathrin coated pits (280). Tyrosine- and dileucine-based motifs represent two sorting signals contained within the cytoplasmic portion of several receptors and transmembrane glycoproteins. Such motifs mediate interactions with either the μ2 subunit of AP-2 to select proteins for inclusion into the vesicle and facilitate their transfer from one part of the cell to another (281,282). Class I PI3Ks have also been shown to be required for the endocytosis of transferrin receptors which in this system occurs directly from the early endosome and independently of the recycling endosome (283).

Similarly, internalisation of the AT_1 angiotensin II (Ang II) GPCR has been shown to occur via multi-vesicular bodies, and recycle to the cell surface by a rapid PI3K-dependent recycling route, in addition to a slower pathway that is less sensitive to PI3K inhibitors (284).

Class II PI3K in the regulation of membrane trafficking

Class II PI3K enzymes include PI3K-C2α, PI3K-C2β and PI3K-C2γ. Little is known about the role of PI3K-C2β and C2γ in receptor sequestration; however the localization and function of C2α is becoming more apparent. PI3K-C2α has been demonstrated to be co-purified with a population of CCVs originating from rat brain homogenates. In addition, the PI3K activity within these CCV preparations was refractory to wortmanin treatment, consistent with the presence of PI3K-C2α. Immunofluorescence analysis revealed that the distribution of PI3K-C2α closely paralleled that of γ-adaptin, a component of the AP-1 adaptor. While CCVs derived from the TGN and plasma membrane both contain clathrin as a major component, the two types can be distinguished by their adaptor protein (AP). Thus, AP-1 is present on vesicles derived from the Golgi apparatus while AP-2 is found on vesicles produced from the plasma membrane. More recently, PI3K-C2α has been shown to play an important role in the formation and un-coating of endocytic vesicles (285,286).

1.20 Aims of the study

It is clear from the above overview that some chemokine receptors have received more attention than others, with the most well documented chemokine receptor system being CXCR4 (25). CXCL12, the ligand for CXCR4, is a highly efficacious lymphocyte chemoattractant known to induce a $G_{\alpha i}$-dependent increase in intracellular free calcium and chemotaxis of CXCR4-expressing cells (255,260,287-290). PI3K has been shown to be required for CXCL12-mediated PKB phosphorylation and chemotaxis, but not for the activation of the MAPK pathway (258,291,292). In contrast, the signalling pathways and function of CCR8 remain ambiguous.

This thesis aims to assess CCR8 receptor signalling pathways. Specifically, the project will investigate 3 main areas as outlined below. The first considers:

- Activation of the MAPK and PKB pathways by CCL1
- Examination of calcium responses and functional chemotaxis assays mediated by CCL1.
- Use of pharmacological inhibitors to identify some of the proteins that are involved within the signalling pathways of interest.

Regulation of signalling pathways is a fundamental process that maintains homeostasis, thus failure to initiate inhibitory mechanisms results in profound immune defects ranging from hyper-proliferation as seen in cancers, to debilitating autoimmunity (212). Two ways in which cells regulate responses are by receptor recycling and the function of lipid phosphatases. This thesis examines both processes in the CXCR4/CXCL12 receptor system. Regulation of PI3K-dependent signalling in B cells is a well characterised model system used to determine the role of the lipid phosphatase SHIP in inhibitory regulation. FcγRIIb-induced recruitment of SHIP has been implicated in the regulation of BCR-mediated responses in B cells (213-215) however the role of SHIP in regulating independently activated receptors, such as chemokine receptors is unknown. The second area therefore aims to:

- Investigate the tyrosine phosphorylation of SHIP following stimulation of the either the BCR, FcγRIIb or CXCR4 receptor.
- Verify the effect of SHIP on BCR-mediated responses.
- Investigate the effect of co-ligation of the BCR and FcγRIIb on CXCL12-mediated responses.
- Utilise a membrane-localised constitutively active SHIP mutant expressed in the Jurkat T cell line (293) using a tetracycline (Tet)-regulated expression system, to verify that SHIP can contribute to the regulation of chemokine-induced PIP_3 accumulation, phosphorylation of PKB/ERK1/2 and chemotaxis.

The second regulatory process, known as chemokine receptor recycling is an important process governing receptor desensitisation and resensitisation. This mechanism not only functions to recycle receptors for further stimulation but also enables cells to downregulate receptor expression in diverting them to the lysosomes for degradation. The receptors are channelled through endosomes which are regulated by enzymes, namely Rab GTPases and PI3Ks. The roles of certain enzymes have been investigated however the β isoform of class II PI3K and the enzyme which regulates the recycling endosome, Rab 11, have not been examined in the CXCR4/CXCL12 receptor system. The objectives of this final part of the study are to:

- Generate an EGFP-CXCR4 construct, to transfect into HEK-293 cells
- Use a GST-AP2β fusion protein to determine whether PI3K-C2β associates with AP2β.
- Investigate the co-localisation of PI3K-C2β with the adaptor AP2β
- Study the co-localisation of PI3K-C2β with enzymes known to be involved in receptor recycling, namely Rab 11a.
- Perform gradient chemotaxis experiments of CXCL12, in order to determine whether PI3K-C2β is involved at the leading edge using the technique of semi-confocal microscopy.

The Hypotheses:

1. CCL1 can induce cell migration and phosphorylation of ERK and PKB.
2. SHIP can modulate CXCR4-mediated responses in B and T cells.
3. CXCR4 is internalised in response to CXCL12 and co-localises with AP2 and PI3K-C2β.

Chapter 2

Chapter 2: Materials

2.1 Antibodies and Plasmids

Antibody	Species	Mono/Polyclonal	Source
F(ab')2 anti-mouse IgG	Goat	Polyclonal	Zymed Laboratories, San Francisco, USA
Anti-mouse IgG	Rabbit	Polyclonal	Zymed Laboratories, San Francisco, USA
Anti-p85α	Mouse	Monoclonal	Doreen Cantrell, School of Life Sciences, Dundee
Anti-p110δ	Rabbit	Polyclonal	Bart Vanhaesebroeck, Ludwig Institute, London
Anti-p110γ (clone H-199)	Goat	Polyclonal	Santa Cruz, California, USA
Anti-SHIP	Goat	Polyclonal	Santa Cruz, California, USA
Anti-hCD3 UCHT1	Mouse	Monoclonal	Doreen Cantrell, School of Life Sciences, Dundee
Anti-mCD3 2C11	Mouse	Monoclonal	Daniel Olive, INSERM, Marseille
Anti-phospho ERK1/2	Rabbit	Polyclonal	NEB, New England, USA
Anti-ERK1/2	Rabbit	Polyclonal	NEB, New England, USA
Anti-phospho PKB^{308}	Rabbit	Polyclonal	NEB, New England, USA
Anti-phospho PKB^{473}	Rabbit	Polyclonal	NEB, New England, USA
Anti-PKB	Goat	Polyclonal	NEB, New England, USA
Anti-phosphotyrosine (4G10)	Mouse	Polyclonal	Upstate Biotechnology, NY, USA

Anti-Mouse HRP	Rabbit	Polyclonal	DAKO, Cambridgeshire, UK
Anti-Goat HRP	Sheep	Polyclonal	DAKO, Cambridgeshire, UK
Anti-Rabbit HRP	Sheep	Polyclonal	DAKO, Cambridgeshire, UK
Anti-PI3K-C2β	Mouse	Monoclonal	BD Biosciences, San Jose, USA
Anti-AP2	Mouse	Monoclonal	Kind Gift from Prof. A. Richmond, Vanderbilt University, Tennessee, USA
Cyanine 3 Fluorophore (596 nm) (Red Dye)	Mouse	Monoclonal	
Cyanine 5 Fluorophore (670 nm) (Far Red Dye)	Rabbit	Polyclonal	
HA-CXCR4			
GFP-CXCR4			
GST-AP2			

2.2 Consumables

ChemoTx disposable chemotaxis chambers	Neuro Probe Inc, Gaithersburg, MD
Conical Centrifuge 15 ml Tubes	BD Biosciences, San Jose, USA
Conical Centrifuge 50 ml Tubes	BD Biosciences, San Jose, USA
Cryovials	Fisher Scientific, Loughborough, UK
Disposable 30 ml Pipettes	NUNC, UK
Disposable 10 ml Pipettes	NUNC, UK
Eppendorf Centrifuge 1.5 ml Tubes	NUNC, UK
Filter Paper (3MM)	Whatman, Maidstone, UK
Flasks 25 cm^2 Tissue Culture	NUNC, UK
Flasks 80 cm^2 Tissue Culture	NUNC, UK

Flasks 175 cm^2 Tissue Culture	NUNC, UK
Gel Loading 100 µl Tips	Fisher Scientific, Loughborough, UK
Gilson Tips 10, 100, 1000 µl	Gilson, USA
Glass Cover Slips	Fisher Scientific, Loughborough, UK
Haemocytometer	Fisher Scientific, Loughborough, UK
MACS LS Column	Miltenyl Biotec, UK
Needles 'Butterfly'	Terumo, Belgium
Nitrocellulose Blotting Membrane	Fisher Scientific, Loughborough, UK
Parafilm	Fisher Scientific, Loughborough, UK
Pastettes 3 ml plastic	Alpha Lab, Hampshire, UK
PCTE-framed 5 µm-pore PVPF filters with adhesive	Porvair Sciences Ltd. UK
Petri Dishes for Tissue Culture	NUNC, UK
Plastic 60 ml Pots with Lids	Sterilin, UK
Polyproylene FACS tubes	BD Biosciences, San Jose, USA
Silica Gel 60 Thin Layer Chromatography Laned Plates	Whatman, Maidstone, UK
Screw-lid Centrifuge 1.5 ml Tubes	Starsted, Germany
Syringes 5, 20, 60 ml	Terumo, Belgium
Syringe Filters for Filter Sterilisation 0.2 µm-pore	Terumo, Belgium
Thin wall 0.2 ml PCR Tubes with lids	Promega, WI, USA
Tissue Culture 6-well plates	NUNC, UK
Tissue Culture 24-well plates	NUNC, UK
Tissue Culture 96-well plates	NUNC, UK
White Opaque 96-well plates	NUNC, UK
X-omat Film	Kodak, Harrow, UK

2.3 Reagents

Acetone	Fisher Chemicals, Loughborough, UK
Acrylamide	Biorad, California, USA
Agarose (Molecular Biology Grade)	Sigma, Poole, UK
Ammonium Phosphate $(NH_4)_2PO_4$	Sigma, Poole, UK
Ampicillin	Sigma, Poole, UK
APS	Sigma, Poole, UK
ATP (100mM aq. Stock (-20°C), dissolved in 100mM Tris pH 7.4	Sigma, Poole, UK
Bovine Serum Albumin (Tissue Culture Grade)	Sigma, Poole, UK
Bovine Foetal Calf Serum (Heat Inactivated)	Life Technologies, Paisley, Scotland
Bromophenol Blue	BDH, Poole, UK
Black 96-Well Plates	Corning Costar, High Wycombe, UK
Butanol	BDH, Poole, UK
Calcein-AM	Molecular Probes, Eugene, OR, USA
Calcium Chloride	Sigma, Poole, UK
Coomassie Blue	Sigma, Poole, UK
Chloroform	BDH, Poole, UK
Cell Titer 96 AQ_{ueous} One Solution Reagent	Promega, WI, USA
Deoxynucleoside Triphosphates: dATP, dCTP, dGTP and dTTP	Boehringer Mannheim, Germany
Dissociation Buffer	Life Technologies, Paisley, Scotland
Digitonin	BDH, Poole, UK

DMSO	Sigma, Poole, UK
DNA 1 kB Ladder	NEB, New England, USA
EDTA	Sigma, Poole, UK
EGTA	Sigma, Poole, UK
ECL	Amersham, Oxfordshire, UK
Ethanol 99.1%	BDH, Poole, UK
Ethidium Bromide	Sigma, Poole, UK
Ethyl Formate	Fisher Chemicals, Loughborough, UK
Flo-Scint IV Scintillation Liquid	Canberra Packard, UK
Folsch Lipids from Bovine Brain	Sigma, Poole, UK
Fluo-4	Molecular Probes, Eugene, OR, USA
Fura-2 AM acetoxymethyl ester	Sigma, Poole, UK
Geneticin (G418)	Life Technologies, Paisley, Scotland
Glacial Acetic Acid	BDH, Poole, UK
Glucose	Fisher Scientific, Loughborough, UK
Glutathione Sepharose Beads	Sigma, Poole, UK
Glutaraldehyde	Sigma, Poole, UK
Glycerol	Fisher Scientific, Loughborough, UK
Glycine	Sigma, Poole, UK
HEPES 1 M Buffer Solution Sterile	Life Technologies, Paisley, Scotland
HBSS (without calcium and magnesium)	Life Technologies, Paisley, Scotland
HCl	BDH, Poole, UK

HPLC Column (Sax Partisphere)	Whatmann, UK
Hygromycin B	Calbiochem, Nottingham, UK
Interleukin-2 (IL-2)	Chemicon, Hampshire, UK
I-309 (CCL1) Chemokine (Recombinant Human)	R&D Systems, Minneapolis, USA
Ionomycin	Calbiochem
IPTG	Sigma, Poole, UK
Kanamycin	Sigma, Poole, UK
Lauryl Sulphate (SDS)	Sigma, Poole, UK
Leupeptin	Sigma, Poole, UK
$LiCl_2$	Sigma, Poole, UK
LB Agar	Sigma, Poole, UK
LB Broth (Miller)	Sigma, Poole, UK
LipofectAMIE Plus Reagent	Invitrogen, Paisley, Scotland
Magnesium Chloride	BDH, Poole, UK
Marvel	Supermarket, UK
Mercaptoethanol	Sigma, Poole, UK
Methanol	BDH, Poole, UK
Methylamine (25-30% in dH_2O)	Fisher Chemicals, Loughborough, UK
Molecular Weight Markers	Life Technologies, Paisley, Scotland
N-Butanol	Fisher Chemicals, Loughborough, UK
NaF	Sigma, Poole, UK
NP-40 1%	Fisher Chemicals, Loughborough, UK
NaCl	Sigma, Poole, UK
Orthophosphoric Acid [^{32}P] 5mCi/ml	NEN, Stevenage, UK

Paraformaldehyde	Sigma, Poole, UK
Phosphate Buffered Saline	Sigma, Poole, UK
Penicillin/Streptomycin	Life Technologies, Paisley, Scotland
Pepstatin	Sigma, Poole, UK
Petroleum Ether (bp 40-60 °C)	BDH, Poole, UK
Phenyl Methyl Sulfonyl Flouride	Sigma, Poole, UK
Phosphoric Acid	Fisher Chemicals, Loughborough, UK
Pluronic Acid	Molecular Probes, Eugene, OR, USA
Phorbol 13-Myristate 12-Acetate	Calbiochem, Nottingham, UK
Poly-L-Lysine	Sigma, Poole, UK
Ponceau S	Sigma, Poole, UK
Potassium Acetate	Sigma, Poole, UK
Probenecid	Sigma, Poole, UK
Propan-2-ol	BDH, Poole, UK
Propan-1-ol	BDH, Poole, UK
Protein A Sepharose	Amersham, Oxfordshire, UK
Protein G Sepharose	Amersham, Oxfordshire, UK
$Pd(N)_6$ random nucleotide hexamers	Boehringer Mannheim, Germany
Potassium Oxalate	Sigma, Poole, UK
Restriction Enzymes (BamHI, Xho-I, HindIII)	Promega, UK
Staphylococcal Enterotoxin B (SEB)	Sigma, Poole, UK
SDF-1 (CXCL12) (Recombinant human)	R&D Systems, Minneapolis, USA
Sodium Hydroxide	BDH, Poole, UK
Sodium Orthovanadate	Sigma, Poole, UK
Sodium Azide	Sigma, Poole, UK
Tetrabutyl Ammonium Hydrogen Sulphate	Sigma, Poole, UK

(TBAS)	
TEMED	Sigma, Poole, UK
Tetracycline	Sigma, Poole, UK
Tris, Hcl	Sigma, Poole, UK
Trypan Blue	Sigma, Poole, UK
Trypsin, EDTA	Life Technologies, Paisley, Scotland
Tween 20	Sigma, Poole, UK
Versene	Life Technologies, Paisley, Scotland

2.4 Cells, Bacteria and Media

BALB/c mouse B lymphoma line, A20	Kind Gift from Dr. D. Cantrell, School of Life Sciences, Dundee
DH5-α Bacteria	Kind Gift from Prof. A. Richmond, Vanderbilt University, Tennessee, USA
HUT-78 Cells	Kind Gift from Prof. J. Westwick, Novartis Pharmaceuticals, Horsham, UK
HEK-293	Kind Gift from Prof. A. Richmond, Vanderbilt University, Tennessee, USA
Jurkat Cells	Kind Gift from Dr. Andres Alcover, Paris, France
Nut Mix F12 (HANS) Glutamax	Life Technologies, Paisley, Scotland
Rat Basophilic Leukaemia CCR8 Stable Transfectants	Kind Gift from Prof. J. Westwick, Novartis Pharmaceuticals, Horsham, UK
RPMI 1640 Culture Medium	Life Technologies, Paisley, Scotland
SHIP clones	Dr. S. Ward, University of Bath, UK

2.5 Kit Assays and Inhibitors

Fluorokine, Human I-309/CCL1 Biotin Conjugate	R&D Systems, Minneapolis, USA
Gel Extraction Kit	Qiagen Ltd, Crawley, UK
QIAquick (Purification of DNA)	Qiagen Ltd, Crawley, UK
Mini Prep Kit	Qiagen Ltd, Crawley, UK
Maxi Prep Kit	Qiagen Ltd, Crawley, UK
LY294002	Calbiochem, Nottingham, UK
RO320432	Calbiochem, Nottingham, UK
PD98059	Calbiochem, Nottingham, UK
U73443	Calbiochem, Nottingham, UK
U73122	Calbiochem, Nottingham, UK
2APB	Calbiochem, Nottingham, UK
Pertussis Toxin	Sigma, Poole, UK

Chapter 3

Chapter 3: TECHNIQUES

3.1 Freezing Down of Cells

Freezing down of cells for long term use is necessary to minimize genetic change and avoid the aging and transformation of cell lines. Firstly, cells were grown to an optimum density, washed and counted. Their viability was checked using trypan blue, a negatively charged chromophore whose activity is dependent on damaged membranes. Cells at a concentration of $1x10^7$/ml were subsequently re-suspended in freezing medium (10% dimethyl sulfoxide, 90% foetal bovine serum (FBS)) and aliquoted into cryovials. To avoid artifacts the cells were frozen slowly (1°C per minute) in a 'MR Frosty' cooler at -80°C. 12 hours later the cryovials were transferred into canes for storage in liquid nitrogen cylinders (294).

3.2 Thawing of Cells

Cells were recovered from liquid nitrogen, thawed immediately in a 37°C water bath and diluted into pre-warmed media. Following gentle mixing, cells were centrifuged, re-suspended in 10 ml of complete medium (20% FBS and 100 U/ml Penicillin and 100 µg/ml Streptomycin) and transferred into a 20 cm^2 flask for overnight culture.

Cells were subjected to visual assessment over subsequent days, then once confluent and recovered, they were transferred into larger tissue culture flasks.

3.3 Cell Culture

This section outlines the methods used to maintain the different types of cells used within this study. All cells were cultured in humidified incubators at 37°C, 5% (v/v) CO_2.

Suspension Cells

The human leukaemic T cell line, HUT-78 (295) were maintained in RPMI-1640, supplemented with 10% heat inactivated FBS, 100 U/ml Penicillin and 100 µg/ml Streptomycin. Cells were grown in suspension in 175 cm^2 tissue culture flasks. Cells were passaged 3 times per week, by splitting each flask 1:5 into the original volume of pre-warmed full growth medium.

Tet-Off Jurkat cells express a membrane-localised constitutively active SHIP mutant and incorporate a tetracycline (Tet)-regulated expression system (293). These clones were maintained in RPMI supplemented with 10% (v/v) heat-inactivated FBS, 100 U/ml Penicillin and 100 µg/ml Streptomycin, 2 ng/ml of tetracycline, geneticin (500 µg/ml), and hygromycin B (300 µg/ml). Cells set-up in preparation for experiments were washed three times in RPMI and seeded at $1x10^5$/ml overnight in complete

media with or without tetracycline (2 ng/ml). Removal of tetracycline from the cell culture for 48 hours induces the expression of the SHIP mutant.

Semi Adherent Cells

The BALB/c mouse B lymphoma line, A20 (296) were maintained in RPMI 1640, supplemented with 10% FBS, 100 U/ml Penicillin and 100 μg/ml Streptomycin and 50 μM β-mercaptoethanol. These cells grew in a semi-adherent manner and adherent cells were harvested before passaging by moderate agitation of the culture flask. The resultant suspension was split 1:3, three times a week.

Adherent Cells

Rat basophilic leukaemia (RBL) (297) CCR8 stable transfectants were maintained in Nut Mix F12 (HANS) Glutamax supplemented with 10% FBS, 100 U/ml Penicillin and 100 μg/ml Streptomycin and 250 μg/ml of geneticin. Confluent cells were removed of media, washed with PBS, overlaid with dissociation buffer and left to incubate at 37°C for approximately 5 minutes. The cells were subsequently dislodged by moderate agitation and dissociation buffer neutralized with complete media. Cells were split 1:3 twice a week.

Human embryonic kidney cells (HEK293) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 100 U/ml Penicillin and 100 μg/ml Streptomycin. HEK293 cells were transfected with the plasmid encoding CXCR4 using lipofectAMINE Plus reagent (described in full later in this section). Cells were harvested by moderate agitation, the resultant suspension was split 1:3, three times a week.

3.4 Transient Transfection of HEK293 cells

Cells at a concentration of 5 x 10^5/ml were plated into Petri dishes containing 6 cover-slips and left overnight to adhere. The medium was replaced the following day with 5 ml of serum-free medium and returned to the incubator until required. Meanwhile, 750 μl of medium was combined with 30 μl of lipofectamine in a 15 ml tube. In a separate 15 ml tube 30 μl of Plus reagent and 5 μg of DNA plasmid were added to an additional 750 μl of medium. Plus reagent is used in conjunction with lipofectamine to enhance transfection in adherent cell lines. Volumes were multiplied up according to the number of plates to be transfected. Tubes were then left to incubate at room temperature (RT) for 15 minutes, mixed together and then left for a further 15 minutes. 1.5 ml of this mix was then added to the cells already containing 5 ml of medium and left in a humidified incubator at 37°C, 5% (v/v) CO_2 for 3 h. Following the 3 h incubation the medium was replaced with complete medium and returned to the incubator. The cells were used 48 h post-transfection.

3.5 Detection of CCR8 using the Fluorokine System

Cells to be stained were pretreated with purified human IgG (10 µl of 1 mg/ml/10^6 cells) for 15 minutes at room temperature in order to block Fc-mediated interactions. In a separate tube, 20 µl of anti-human CCL1 antibody was mixed with 10 µl of biotinylated CCL1/I-309 and allowed to incubate for 15 minutes at room temperature. In a volume of no more than 25 µl, $1x10^5$ Fc-blocked cells were subsequently added to this mixture. A negative staining control was also set up, consisting of 10 µl of negative control reagent (protein biotinylated to the same degree as CCL1) mixed with an identical sample of cells. All samples were left to incubate for 60 minutes at 4°C. 10 µl of avidin-FITC reagent was then added to each tube and incubated for a further 30 minutes at 4°C in the dark. Finally the cells were washed twice with 2 ml of 1X cell wash buffer (buffered saline-protein solution) to remove un-reacted avidin-fluorescein and resuspended in 200 µl of 1X wash buffer for cytometric analysis.

3.6 Cell Stimulations

Cells were harvested according to the cell culture protocol. All cell lines were pelleted by centrifugation at 1840 x g at room temperature. Cell pellets were pooled and subsequently washed 3 times in serum-free media. Finally cells were assessed for viability, counted with a haemocytometer and resuspended in the appropriate volume

of 0.1% BSA serum-free medium. The suspension of cells were then aliquoted in 0.5 ml volumes into 1.5 ml eppendorf tubes and left to equilibrate for 30 minutes in a 37°C water bath prior to stimulation.

Cells were stimulated with agonist at the required concentration for the specified time. Reactions were terminated by pelleting at 4,500 x g for 10 seconds, removing supernatant and adding 500 µl of ice-cold lysis buffer (appendix 1). Samples were subsequently vortexed and rotated at 4°C for 20 minutes. Finally, cell debris was removed by spinning in a cold microfuge, at 15,000 x g for 10 minutes, and supernatants transferred to clean eppendorf tubes.

Whole Cell Lysates

Sample buffer was added to the supernatants following the removal of cell debris. Whole cell lysates were subsequently boiled for 5 mins on a hot block prior to running on SDS-PAGE.

Immunoprecipitation from Cell Lysates

Cell lysates were pre-absorbed with 20 µl of a 50:50 protein-A/G sepharose slurry for 30 minutes on a rotator at 4°C. Samples were then spun at 15,000 x g to pellet the sepharose beads and supernatants transferred to clean eppendorf tubes. Lysates were

then incubated with 2-5 μg of antibody and rotated for 2-4 h at 4°C. Then, 20 μl of a 50:50 pre-washed proteinA/G sepharose slurry was added to the lysates and incubated for a further 1-2 h on the rotator. For immunoblotting, beads were then washed three times in ice-cold lysis buffer and resuspended in 10-20 μl of sample buffer and boiled for 5 minutes.

3.7 Western Blotting

SDS-PAGE

Immunoblot analysis requires the separation of isolated cellular proteins according to size by SDS polyacrylamide gel electrophoresis. The apparatus used was the Bio-Rad mini-protean-III gel equipment, which was assembled according to the manufacturer's guidelines. The resolving gel was prepared at the desired percentage (appendix 1), poured between the two glass plates and overlaid with water. Once polymerisation had occurred the water was aspirated and the stacking gel was poured (appendix 1). A 15-well comb was inserted to create the wells for the loading of the protein samples. Once set, the comb was removed and the gel was placed into a gel tank containing 1X SDS-PAGE running buffer (appendix 1). The protein samples were run through the stacking gel at 80 V and the resolving gel at 170 V until the bromophenol blue dye front had reached the bottom of the gel. The gel was subsequently removed from the apparatus and soaked in semi-dry transfer buffer

(appendix 1). The separated proteins were transferred from the gel and immobilised onto nitrocellulose by semi-dry transfer using Pharmacia-Biotech Multiphor II electro-blotting equipment. Upon the bottom (positive) of the two graphite electrodes a stack was made consisting of 4 pieces of gel-sized 3MM Whatman paper, a piece of nitrocellulose membrane, the gel and another 4 pieces of 3MM Whatman paper (all soaked in the transfer buffer). Any air bubbles were expelled before the upper (negative) electrode, dampened with semi-dry transfer buffer, was gently lowered into place. The gel was transferred for 60 min at a constant 0.8 mA per cm^2 of membrane.

Immunostaining

Following transfer, blots were rinsed in TBS washing buffer (appendix 1) and then blocked overnight in 1% BSA TBS + 0.1% azide. Primary antibodies were diluted 1 in 1000 in TBS + 0.1% azide. Primary antibodies were applied for 3-4 h at room temperature or overnight at 4°C depending on the antibody used. Blots were subsequently washed 4 times with TBST (appendix 1) for 10 minutes each wash. Antibody bound proteins were detected using a secondary antibody bound to horseradish peroxidase (HRP), which was relevant to the species in which the primary antibody had been raised. Secondary antibodies were diluted between 1 in 10,000 and 1 in 20,000 in 0.5% powdered milk (marvel) TBST depending on the antibody and applied for a minimum of 1 h. Membranes were then washed twice in

TBST and twice in TBS (10 mins per wash) and incubated for 1 minute in enhanced chemiluminescence (ECL) reagent. Reactive protein bands were visualised by exposure of the membrane to Kodak X-AR5 film, followed by developing with the RGII Fuji X-ray film developer.

3.8 Stripping of Membranes

Membranes were submerged in stripping buffer (appendix 1) and incubated in a 60°C water bath for 30 minutes. Following rigorous washing with TBST, membranes were blocked overnight in 1% BSA TBST. Immunostaining was then carried out as previously described.

3.9 Calcium Mobilization

The following section describes the methods used to investigate the calcium mobilisation of adherent and suspension cells. Using either of the calcium-sensitive fluorochromes Fura-2 or Fluo-4 AM, intracellular [Ca^{2+}] can be readily measured. Both Fura-2 and Fluo-4 are coupled to the acetoxymethyester (AM) which permits entry into cells. Following entry, AM is cleaved by endogenous esterases, preventing the escape of the fluorochrome from the cells. On binding to calcium ions, the fluorescence excitation maximum of the fluorochrome transfers to a lower

wavelength without any alteration in the emission spectrum, thereby enabling Fura-2 and Fluo-4 to be used as a dual excitation indicator.

Fura-2 (AM) is ratiometric and ultra-violet (UV) light excitable, with an excitation ratio of 380/340 nm (free-calcium/bound-calcium). Ratioing considerably reduces the effects of uneven dye loading, leakage of dye and photobleaching. Fluo-4 (AM) has an excitation wavelength of 488 nm by argon-ion laser sources, ideal for confocal laser-scanning microscopy.

Adherent cells – FLIPR 96

RBL–CCR8 cells at $3x10^5$/ml in growth medium were plated in clear, flat-bottom black-walled 96-well plates. The flat bottom ensures that the cellular fluorescence is localised to a single horizontal plane, while the black-walls prevent well-to-well crosstalk. Plates were incubated at 37°C / 5% CO_2 for 24 h to allow the cells to recover. Cells were subsequently washed with Assay Buffer (HBSS/ 20 mM HEPES (without Phenol Red indicator) + 0.1% BSA w/v), resuspended in 100 µl of Loading Buffer (22 ml of Assay Buffer supplemented with Fluo-4, probenecid (2.5 mM final) and Brilliant Black (100 µM final)) and left to incubate for 30 minutes at 37°C / 5% CO_2. The anion exchange protein inhibitor, probenecid, was used to prevent the extrusion of Fluo-4 from the cells by organic ion transporters. Washing of the cells following incubation with Fluo-4 was not necessary due to the presence of brilliant black in the assay buffer. The PLC inhibitor (U73122) was added along with the

Fluo-4 whilst the PTX was added 4 h prior to the start of the experiment. Chemokines were aliquoted in a separate 96-well plate at 3X the required concentration to compensate for the dilution factor experienced on addition to the cell plate. The extent of calcium mobilisation was determined using a 96-well fluorescent imaging plate reader (FLIPR-96).

Raw data from the FLIPR was analysed using FLIPR software to produce the minimum and maximum fluorescence for each well (Figure 14). The data was then normalised for cell number using the following formulae:

$$\textbf{Normalised fluorescence} = \frac{\textbf{Fluorescence maximum} - \textbf{Fluorescence minimum}}{\textbf{Fluorescence minimum}}$$

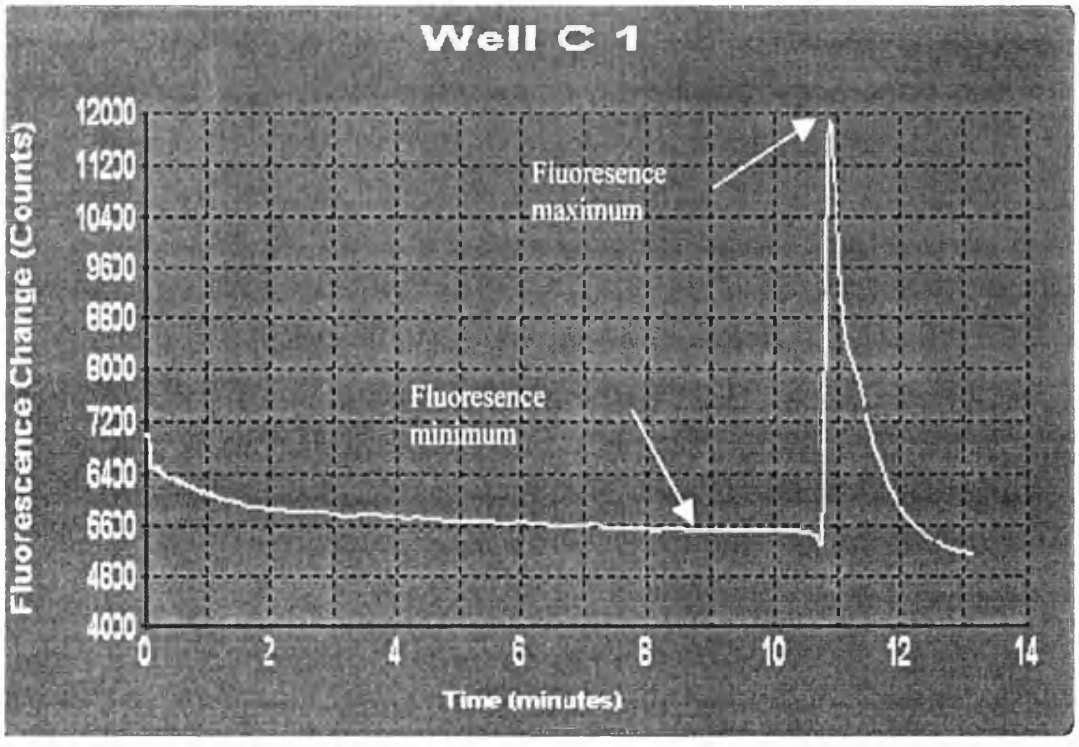

Figure 14. Determination of Minimum and Maximum Fluorescence

Suspension Cells – Flow cytometric analysis

The fluorimeter was first calibrated by monitoring the fluorescent changes in cell suspensions following the addition of 0.16 μg/ml digitonin (to lyse cell membranes and expel intracellular calcium). Fluorescence was monitored using a dual excitation/dual emission spectrofluorimeter (Photon Technologies) for 30 seconds followed by the addition of 40 mM sodium hydroxide (NaOH) and 4 mM EGTA. Conversion of the fluorescence ratios at two wavelengths into intracellular calcium concentration was achieved using the equation developed by Grynkiewicz *et al* (298).

$$[Ca^{2+}]_i = Kd \frac{R-R_{min}}{R_{max}-R} \frac{S_{f2}}{S_{b2}}$$

Where,

- Kd = effective dissociation constant for Fura-2 (2.24×10^{-7} M)
- R = Ca^{2+} bound/ Ca^{2+} free fluorescence ratio
- R_{min} = fluorescence ratio with zero calcium
- R_{max} = fluorescence ratio under saturating conditions
- S_{f2}/S_{b2} = ratio of fluorescence values for Ca^{2+}/ Ca^{2+} free indicator measured at the wavelength used to monitor Ca^{2+} - free indicator (denominator wavelength of R) i.e. at 380 nm.

Intracellular free calcium concentration ($[Ca^{2+}]_i$) of A20 cells was determined by pre-loading the cells ($2x10^6$/ml/sample) with 5 μM of Fura-2 AM at 37°C for 30 minutes.

Cells were washed free of excess Fura-2 using HBSS buffer supplemented with 100 μM Ca^{2+} and 1 mM Mg^{2+} and re-suspended at $2x10^6$/ml. Fura-loaded cells were aliquoted into a 2-ml cuvette. To the cell suspension 20 μl of a 100 mM calcium chloride stock (final [1 mM]) was added and allowed to equilibrate at 37°C in the spectrofluorimeter for 5 minutes. Prior to the addition of the agonists, a basal calcium measurement was taken for 30 seconds. The agonist-induced response was monitored for at least 500 seconds and was detected on the spectrofluorimeter (Photon Technologies).

3.10 Chemotaxis Assays

Two methods of the chemotaxis assay were used in this thesis.

ChemoTx Chamber

The transmigration of cells in response to chemokine was determined using a disposable ChemoTx chamber consisting of a 96-well plate, a hydrophobic polycarbonate filter (5 μm-pore size) and an attachable lid (Figure 15). Cells were washed twice with RPMI and re-suspended in 0.1% BSA RPMI at $4x10^7$/ml. $1x10^7$ cells were then loaded with 5 μg of Calcein-AM on a rotator for 30 minutes at 37°C. Following incubation, cells were washed twice with RPMI and re-suspended at the

required concentration in 0.1% BSA RPMI. The bottom wells of the plate were carefully loaded with 29 µl of agonist, avoiding the formation of air bubbles, and then overlaid with a hydrophobic polycarbonate filter (5 µm poresize). 25 µl of calcein-loaded cells were subsequently aliquoted on top of the filter, covered with a lid, and incubated for 90 minutes in a 37°C incubator. Following incubation, the non-migrated cells were removed from the top of the filter using PBS. The cells that had migrated through the filter were assessed by recording the fluorescence signal using a Fluoroscan II plate reader at 485 nm excitation and 538 nm emission using the bottom reading mode.

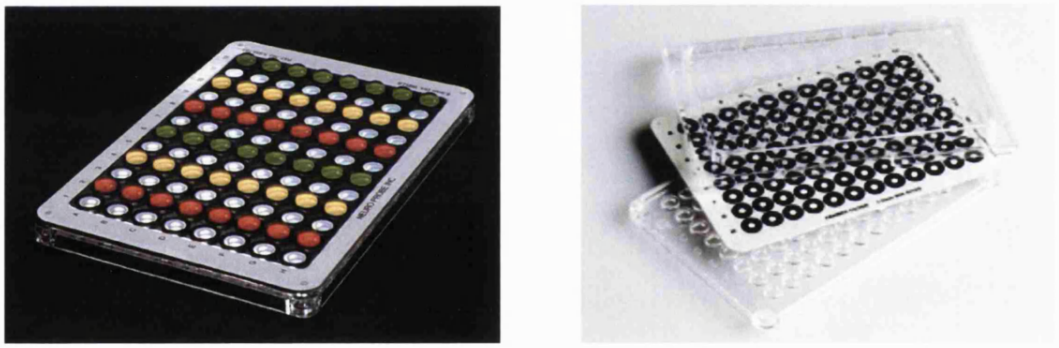

Figure 15. Neuroprobe ChemoTx Disposable Chamber (ref.www.neuroprobe.com)

Boyden Chamber

Chemotaxis was examined using a Boyden 96-well chemotaxis chamber (Figure 16). Cells were washed twice in serum-free RPMI 1640 and resuspended at a density of $1x10^6$cells/ml in RPMI with 0.1% (w/v) BSA. Cells were serum-starved for 1 hour at 37°C. During the starvation period, a 96-well plate was carefully positioned into the chemotaxis chamber and loaded with 395 µl chemokine (0-1 nM final concentration) so as to form a positive meniscus within the well. A 5 µm filter was lowered on top of the 96-well plate, taking great care not to form air bubbles. The gasket was positioned onto pins on the underside of the chamber lid and the lid gently closed, applying even pressure to the top of the chamber. $1x10^6$cells/ml were then added to the wells of the chemotaxis chamber, covered with parafilm, and incubated in a humidified cell incubator at 37°C for 1.5 h. Some cells were retained and serially diluted to give a standard curve ranging from $2x10^5$ to $1x10^3$cells/100 µl/well in a 96-well plate, which was also incubated at 37°C. At the end of the incubation period, any remaining cells were aspirated from the upper wells of the chamber, 200 µl of versene was added per well and the chamber incubated at 4°C for 20 minutes. After aspirating off the versene, the chamber was carefully opened and the 96-well plate removed, without detaching the filter on top. The plate together with the filter was spun at 1511 x g for 10 minutes in a Beckman GS-15R centrifuge (S2096 rotor) to pellet migrated cells. Cells were resuspended in 100 µl RPMI and 20 µl/well cell titre reagent added to both the chemoataxis and standard curve plates. Plates were incubated at 37°C for a

further 1-2 h, after which the absorbance was measured at 490 nm in a 96-well microplate reader (Dynatech MR5000). The number of migrated cells was calculated from the standard curve prepared using the known cell densities.

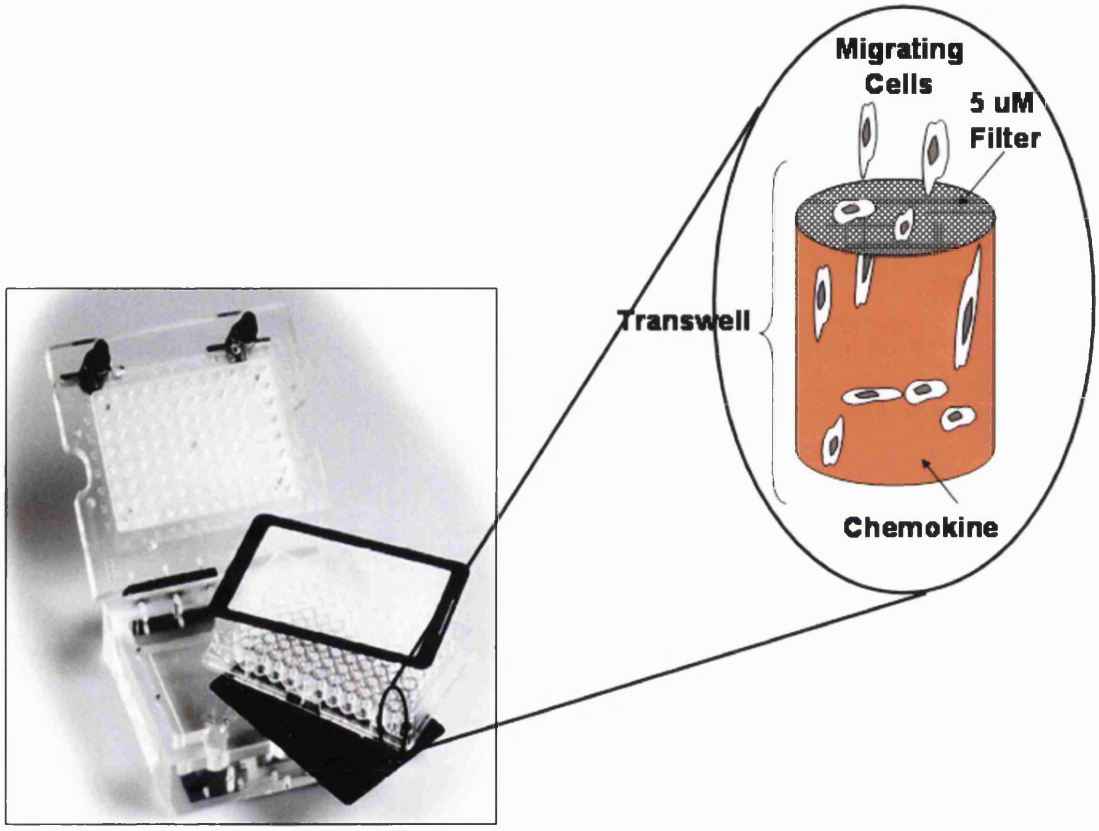

Figure 16. MBB96 Boyden Chamber (ref. www.neuroprobe.com)

3.11 Chemotactic Index

ChemTx Assay:

The chemotactic index was calculated by dividing the mean fluorescence signal of migrated cells in chemokine-containing wells by the mean fluorescence signal of cells that migrated spontaneously toward media alone.

Boyden Chamber Assay:

The chemotactic index was calculated by dividing the number of migrated cells in chemokine-containing wells by the number of cells that migrated spontaneously toward media alone.

3.12 Statistics

Statistical analysis was performed on mean intra-experimental values from 3 or more experiments using a Student t test. For multiple comparisons, the significance was determined using the Student t test with Bonferroni's correction for multiple comparisons. In all chemotaxis experiments data are presented as mean ± SEM for n = 3 replicates.

3.13 Measurement of D-3 Phosphoinositide Lipids in A20 cells

In order to measure D-3 inositol lipid accumulation, cells must be depleted of phosphate prior to labelling by washing three times in 50 ml phosphate-free balanced salt solution, incubating for 10 minutes at 37°C between washes. Cells were resuspended in 10 ml of balanced salt solution supplemented with 5% dialyzed FCS and 20 mM HEPES. 1 mCi of [^{32}P]Pi was added to the suspension of cells and incubated at 37°C for 2 h. After incubation at 37°C, cells were washed 3 times in 50 ml of phosphate free media to remove any unincorporated [^{32}P]Pi and resuspended at a concentration of $2x10^7$ cells/ml in a final volume of 120 µl per point. Cells were stimulated as normal and reactions terminated by addition of 500 µl chloroform/methanol/H_20 (32.6/65.3/2.1 v/v) to produce a homogeneous primary extraction phase. Phases were separated by addition of 200 µl chloroform containing 10 µg/ml Folsch lipids and 200 µl of 2.4 M HCl, 5 mM tetrabutylammonium sulphate followed by vortexing and centrifugation at 1000 x g for 5 minutes. Lower phases were harvested into fresh tubes containing 400 µl of 1 M HCl, 5 mM EDTA, vortexed and centrifuged for a further 5 minutes at 1000 x g. Lower phases were once again removed into fresh tubes and dried *in vacuo*. The extracted lipids were then subjected to a deacylation step to remove the sugar backbone from the lipid. This was achieved by the addition of 1 ml 25% (w/v) methylamine/methanol/N-butanol (4:4:1) followed by incubation at 53°C for 40 minutes. Samples are then cooled rapidly on ice for 5 minutes and dried *in vacuo*

overnight. Samples were then resuspended in 0.5 ml H_20 followed by the addition of 0.6 ml N-butanol/petroleum ether/ethyl formate (20/4/1 v/v), vortexed and centrifuged for 5 minutes at 1000 x g. The upper organic phase was removed and discarded whilst the lower was washed with a further addition of 0.6 ml of butan-1-ol/petroleum ether/ethyl formate mix. After vortexing, centrifugation and removal of the upper phase as before, the lower phase was dried *in vacuo.* Finally pellets were re-dissolved in 90 µl H_20 and analysed by HPLC using a 12.5 cm Whatman Partisphere SAX column (299).

3.14 GFP-CXCR4 Construct

The following steps were carried out to make green fluorescent protein (GFP) tagged CXCR4-expressing HEK-293 cells.

Polymerase Chain Reaction (PCR)

PCR is an *in vitro* technique, where a given DNA sequence is identically copied. The number of copies rises exponentially, since every newly synthesized DNA-sequence is also a matrix for the next copy. The PCR-reaction is subdivided into three steps:

1. Denaturation

During denaturation, the template-DNA is separated (denaturated) into its two separate strands by heating up the temperature to 95°C.

2. Annealing

The temperature is lowered to a degree specific for the primer (oligonucleotide), which generally lies between 50°C and 70°C. This guarantees, that the primer takes its place at the specific DNA-recognition-sequence of the single-stranded DNA-template-sequence.

3. Synthesizing

The third step, the sythesizing, takes place at a temperature of 71°C degrees. This corresponds to the optimal temperature for the Taq-polymerase enzyme to work. The polymerase ensuingly prologues the paired short oligonucleotides according to the DNA-matrix, until the double-stranded DNA-molecule is complete again.

PCR Primers

The dried oligonucleotides (primers) (200 nM) were first diluted to 25 pM with dH_20, and subsequently added to the PCR Master Mixtures detailed in appendix 1. The master mixtures were mixed and run on a PCR machine overnight. The temperatures

set up on the machine were 95°C, followed by 51°C, followed by 71°C, each for 30 cycles.

Purification of PCR product

Sequencing of the PCR product was not necessary because the template used for the PCR was a CXCR4 plasmid previously sequenced by Prof. Ann Richmond from Vanderbilt University. However, a sample of PCR product was run on a 1% agarose gel containing 5 µl of a 10% stock of ethidium bromide solution to check for dDNA (CXCR4). The gel was run in TAE buffer (appendix 1) at 100 V.

The PCR product was then purified from the reaction mixture using a QIAquick PCR Purification kit (QIAGEN) according to the manufacturer's protocol. In summary, 250 µl PB buffer was added to 50 µl of PCR product, and filtered through a mini-column by spinning the tube for 1 minute at 16100 x g. The supernatant was discarded and a further 750 µl of PE buffer was added to wash the DNA trapped on the filter. The tube was spun again and supernatant discarded. The column was subsequently transferred to a fresh tube and eluted with the addition of 30 µl of dH_20 and spun once more.

Digestion of PCR product and EGFP-N1 Vector

The next step was to digest the PCR product and EGFP-N1 vector using 2 restriction enzymes (Promega) (Figure 17). The buffers used were matched according to efficiency data provided by the company data sheet. In this case, both restriction enzymes XhoI and HindIII gave maximal efficiency when using buffer B. Digestion Mixtures were made up as described in appendix 1. The mixtures were briefly centrifuged and incubated in a 37°C water bath for 3-4 h. The digested vector and DNA were subsequently run on a 1% agarose gel to separate the ligated vector from the target gene insertion site and the CXCR4 sequence from the extra oligonucleotides.

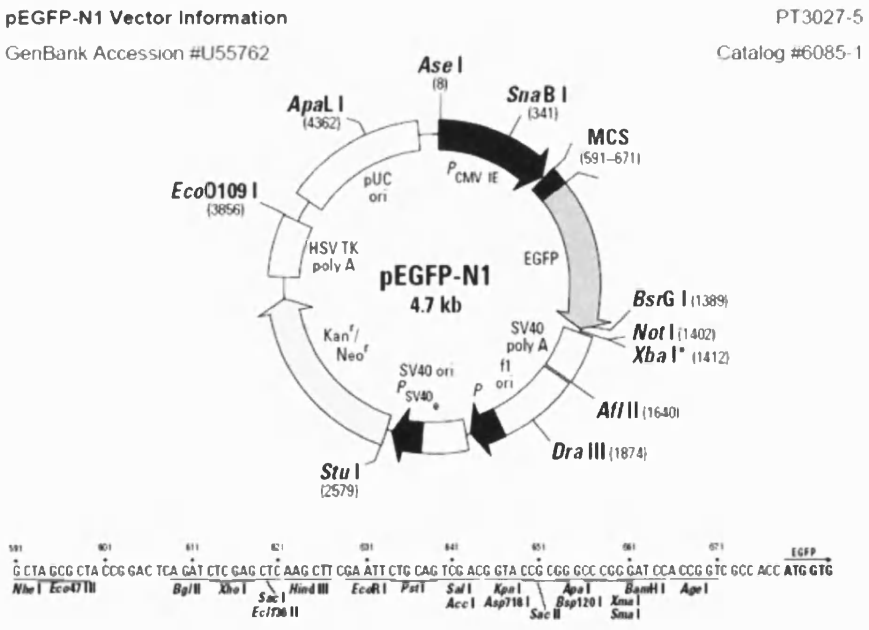

Figure 17. pEGFP-N1 Vector Information

Gel Purification

The identified DNA bands for CXCR4 and the vector were cut from the gel and placed into separate eppendorf tubes. DNA fragments were purified from agarose gels using the QIAquick gel extraction kit (QIAGEN) according to the manufacturer's protocol. In summary, the weight of the gel within each tube was determined and 3 times the volume of QG buffer was added. The gel was melted by incubating the tubes for 10 minutes at approximately 50°C. The solutions were then added to Eppendorf columns, centrifuged and washed with PE buffer. The columns were finally transferred to fresh tubes and plasmid DNA eluted with 20 µl of dH_2O.

Ligation Step

Two ligation mixtures were set-up containing different ratios of vector plasmid:DNA insert, a 1:2 and 1:3. 20 µl volumes of ligation mixtures contained 2 µl 1X T_4 DNA ligase buffer, 4 or 6 µl DNA insert, 2 µl DNA vector, 1 µl of 1X T_4 DNA ligase and 11 or 9 µl dH_2O. The ligation mixtures were then left at room temperature for 2 h.

Transformation into Bacteria

Two tubes were set up with 100 µl of DH5-α bacteria containing either 4 µl of plasmid from tube 1, or 4 µl from tube 2. The bacteria were then placed on ice for 10 minutes, transferred to a 42°C water bath for 50 seconds and returned to ice for a further 2 minutes. The bacteria were then spread on agar plates containing kanamycin and incubated overnight at 37°C.

Purification of Plasmid DNA

A single colony was selected from the kanamycin resistant plates and added to 3 ml of LB broth, labelled and incubated overnight. At least 8 colonies were selected at any one time.

Mini Prep using a Qiaquick kit

1.5 ml of bacteria from the mini culture were transferred into an eppendorf tube, labelled and centrifuged for 5 minutes at 16100 x g. The supernatant was discarded and the pellet gently resuspended in 250 µl of P1 buffer. 250 µl of P2 buffer (lysis buffer) was then added and mixed followed by 350 µl of P3 buffer (neutralization buffer). The cell debris was removed by centrifuging for 10 minutes, and supernatants

transferred to the top of a fresh tube containing a column insert. This was then centrifuged for 1 minute, supernatant removed and column washed with 750 µl of PE buffer. Finally 16 µl of dH_2O was added to elute the plasmid.

3.15 Digestion of plasmid DNA

To purify the plasmid DNA the Digestion Reaction Mixture was made up as described in appendix 1. The above reaction mixtures were left to digest in a 37°C water bath for 1 h, then run on a 1% agarose gel to separate the 1 kb CXCR4 from the 4.7 kb vector. The bands were cut from the gel and purified using the gel extraction kit as described above.

Maxi Prep of positive clones

200 µl of bacteria (positive clone identified from the mini prep) were incubated overnight in a conical flask containing 200 ml of LB broth and 100 µg/ml of kanamycin.

Purification of Plasmid from Maxi Prep

The majority of bacteria from the mini culture were transferred into large centrifuge bottles, labelled and centrifuged for 10 minutes at 6000 x g. The supernatant was discarded and the pellet gently resuspended in 10 ml of P1 resuspension buffer. 10 ml of P2 buffer (lysis buffer) was then added and mixed followed by 10 ml of P3 buffer (neutralization buffer). The mixture was then poured into the barrel of a QIA filter cartridge and left to stand for 10 minutes at room temperature. Meanwhile the columns were equilibrated and washed with QBT buffer. The DNA was then eluted by adding 15 ml of elution buffer and precipitated with 10.5 ml of isopropanol. The DNA was then centrifuged at 20,000 x g for 30 minutes at 4°C, supernatant removed and pellet washed with 70% ethanol. The pellet was air dried and dissolved in 250 µl of dH_2O.

Digestion of Plasmid DNA

Two separate digests were then carried out to ensure the plasmid DNA was CXCR4. The first contained the restriction enzymes XhoI and HindIII and the second, BamHI and XhoI. The protocol was followed as described above for the digestion and gel purification of the PCR product and vector.

3.16 Preparation of GST Fusion Proteins

1 µl of GST-AP2 or 1 µl of GST alone was added to two separate tubes containing 100 µl of bacteria. The transformation of bacteria was carried out as described above, however bacteria were spread onto agar plates containing ampicillin not kanamycin. The mini prep and maxi prep were also followed as described above. The fusion protein was then transfected into HEK-293 cells as previously described.

GST-AP2 Purification

This part of the experiment was carried out entirely on ice. The bacteria from the maxi prep were centrifuged for 10 minutes at 400 x g and lysed with 2 ml of lysis buffer containing 100 µg/ml of PMSF. The bacteria were resuspended, sonicated, transferred to eppendorf tubes and centrifuged for 10 minutes at 4°C. The recovered protein was pooled and the concentration determined by Bradford assay (Biorad). Using 10 µl of protein, serial dilutions in water were made and added to 50 µl Bradford reagent. Protein concentration was determined by optical density readings at 595 nm and comparison to a range of known protein standards (2 mg/ml – 0.05 mg/ml). Glutathione beads were then washed to remove the ethanol using lysis buffer and added (200 µl) to GST-AP2 and GST containing tubes. Protein complexes were adsorbed through the addition of 30 µl GST sepharose slurry, and rotation for 1 h at

4°C. Adsorbed proteins were recovered via sedimentation of the sepharose beads by centrifugation, followed by 5 washes with lysis buffer. Finally, beads were sedimented and drained with a Hamilton syringe, prior to boiling in 30 µl SDS-Laemmli sample buffer.

Incubation of lysates with GST alone or GST-AP2

Lysates were prepared as described previously and incubated overnight with either GST-AP2 or GST alone at 4°C. Samples were subsequently washed in lysis buffer, followed by the addition of 35 µl of sample buffer and boiled for 5 minutes. Gradient gels were set-up and samples run as previously described for SDS PAGE and Western Blotting.

3.17 Co-localisation Assay by Confocal Microscopy

Cells were plated at $5x10^5$/ml in 10 ml of complete media in the presence of 6 coverslips (0.1% glycine treated) and incubated overnight. Cells were stimulated with ligand for the indicated time points and fixed in methanol. Cells were washed in PBS, incubated with primary anitbody (1:50) for 30 minutes followed by secondary antibody (1:1000) for 30 minutes. The cells were washed in PBS, inverted onto slides using mounting media and viewed by confocal microscopy.

3.18 Co-localisation during Chemotaxis

Parental cells were plated at $5x10^5$/ml with coverslips and incubated overnight. The following day the cells were transfected with CXCR4 as described previously. Prior to stimulations, coverslips were clipped with tweezers to note the direction of chemokine. A lid of a Petri dish was adapted with a filtered well to allow the chemokine to be released in a gradient manner (Figure 18). Stimulations were carried out as described in the co-localisation assay, followed by incubations with antibodies specific for $pPKB^{308}$ and PI3K-C2β. Cells were subsequently analysed on a semi-confocal/convoluting microscope.

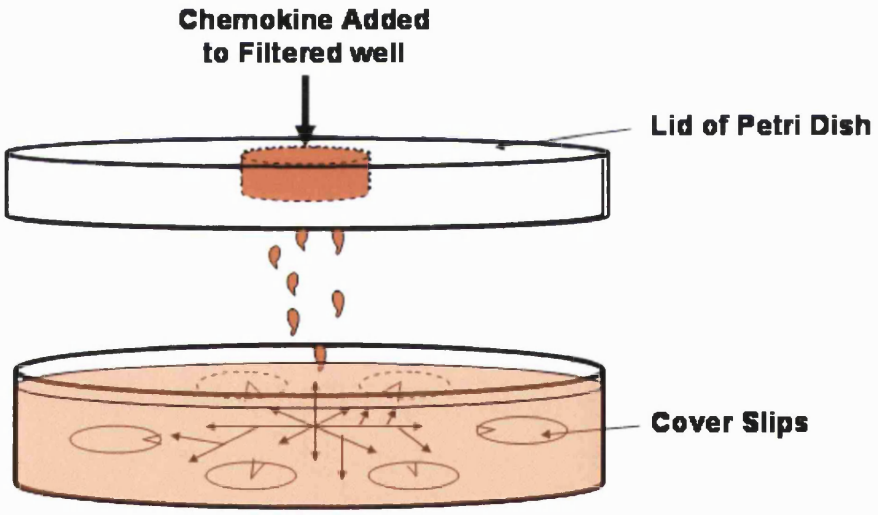

Figure 18. The adaptation of a Petri dish for a gradient chemotaxis assay.

A hole was made in the lid of the Petri dish with a filtered well glued to it, to allow the addition and slow release of chemokine. The triangles in the cover slips represent the direction of the chemokine gradient.

The following chapters provide the results of the experiments carried out.

Chapter 4

Chapter 4

Results: Signalling Events mediated by CCL1/CCR8 ligand-receptor pair

4.1 Introduction

Many studies have investigated possible roles for CCR8 however the exact intracellular signalling pathways involved remain unknown. Research into the chemokine receptor CCR8 and its respective ligand CCL1 has been sparse as outlined in Chapter 1. This chapter provides the results of experiments which aim to identify the effectors and reveal the pathways and functional responses mediated following the ligation of CCR8 by CCL1 in transfected cell models and leukaemic T cell lines.

The ERK1/2 and PKB signalling pathways regulate cell differentiation, proliferation and cell survival, and are two of the best characterised signalling cascades. Both pathways have been demonstrated to be activated downstream of chemokine receptors in a variety of situations (287,300). However, activation of these cascades in response to CCR8 ligation has yet to be determined. This study aims to establish and define the activation of these signalling modules using two different cell systems and compare them to previously reported studies. These include the adherent rat basophillic leukaemia cells (RBLs) stably transfected with CCR8, and the leukaemic T cell line HUT-78. RBL cells were chosen because they do not endogenously express CCR8. This permits the authenticity of the CCL1/CCR8-mediated response to be verified. Unfortunately, the coupling of receptors to downstream biochemical events may not accurately reflect the situation in physiological conditions. Therefore

HUT-78 cells which endogenously express CCR8 were additionally used to dissect CCL1-mediated signalling cascades. The fact that HUT-78 cells are a suspension cell line enabled a technically more straightforward investigation of CCL1-mediated directed T cell migration.

4.2 Analysis of CCR8 Signal Transduction in RBL Cells

Surface Expression of CCR8 on RBL Cells.

The expression of CCR8 was investigated on RBL cells stably transfected with CCR8. Rabbit polyclonal antibodies against human CCR8 have been developed by Alexis, abcam, Imgenex and Exalpha biologicals; however none of these were able to detect the expression of hCCR8 on RBL-CCR8 cells by FACs analysis *(Brown Z. Novartis Pharmaceuticals, personal communication).* As an alternative approach the expression levels of CCR8 were determined indirectly using a kit which relies on the binding of biotinylated chemokine (CCL1) and avidin-fluorescein (See chapter 3 for more details). Cells expressing the specific chemokine receptor (CCR8) are fluorescently stained, with the intensity of staining being proportional to the density of the receptor. Relative receptor density is then determined by flow cytometric analysis using 488 nm wavelength laser excitation. The histogram represents analysis of 10,000 events acquired in the total population (Figure 19).

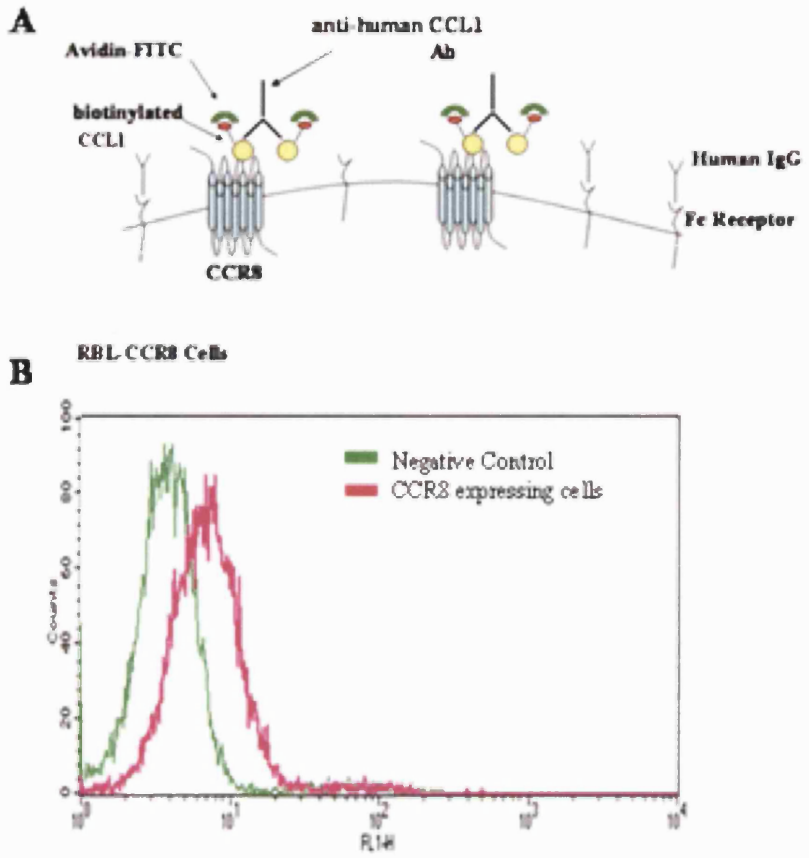

Figure 19. CCR8 expression on RBL-CCR8 Cells

(A) CCR8 expression was detected using the 'Fluorokine' kit from R&D. (B) Cells were first pre-treated with human IgG to reduce non-specific binding (10 µl of 1 mg/ml stock/10^6 cells) for 15 minutes at RT. 1 x 10^5 cells were then incubated with a pre-mixed cocktail of 20 µl of anti-human CCL1 antibody and 10 µl of biotinylated CCL1 (The antibody functions to amplify the signal). The green line corresponds to the negative control (a protein biotinylated to the same degree as CCL1) and the pink line corresponds to an increase in fluorescence demonstrating the positive expression of CCR8 on these cells. Data are from a single experiment representative of 2 others.

Use of PTX to Elucidate the $G_{\alpha i}$ dependency of CCL1-mediated Ca^{2+} Mobilization in RBL-CCR8 Cells

A common indicator of biochemically functional chemokine receptors is the mobilisation of intracellular calcium. Chemokines are mainly known to signal through seven-transmembrane GPCR (301) that activate a $G_{\alpha i}$ protein. $G_{\alpha i}$ association with several chemokine receptors has been described (302,303); most responses to chemokines are therefore inhibited by the $G_{\alpha i}$-specific inhibitor, PTX, although there are examples of PTX-insensitive G proteins being coupled to certain chemokine receptors (e.g. $G_{\alpha q}$ couples to CCR2 in HEK-293 cells) (304). PTX catalyzes the ADP-ribosylation of the α subunits of the heterotrimeric regulatory G proteins. This prevents the G protein heterotrimers from interacting with receptors, thus blocking their coupling and activation. Experiments were carried out to determine whether CCL1-mediated calcium mobilisation was reliant on the coupling of $G_{\alpha i}$ to CCR8. CCR8-transfected RBL cells demonstrate a concentration dependent calcium response to CCL1 as indicated by the intensity of Fluo-4 (Figure 20). Pre-incubation of these cells with PTX (100 ng/ml) for 4 h prevented CCL1, at all concentrations, from inducing a Ca^{2+} response.

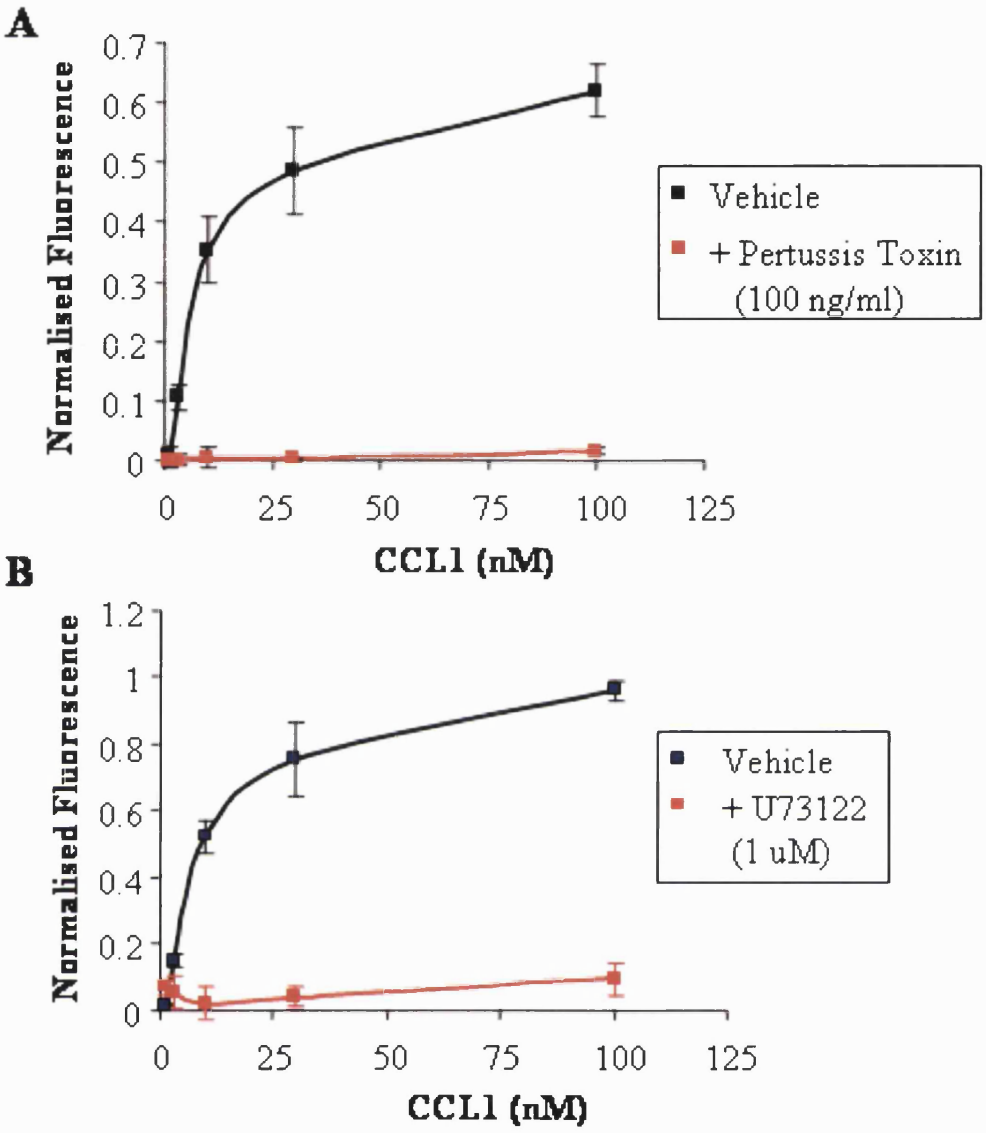

Figure 20 CCL1 mediates a $G_{\alpha i}$ and PLC-dependent Ca^{2+} Mobilization in RBL-CCR8 Cells.

RBL cells were aliquoted in a 96-well plate at $3x10^5$/ml in complete media and incubated overnight to allow cells to adhere. Cells were pre-incubated with vehicle or with either (A) PTX (100 ng/ml) for 4 h prior to loading with Fluo-4 (30 minutes) or (B) pre-incubated with the PLC inhibitor, U73122 (1 μM) at the same time as Fluo-4 (30 minutes). Cells were subsequently stimulated with CCL1 at concentrations between 1 and 100 nM and analysed using the FLIPR-96 in association with FLIPR software as described in materials and methods. Data are from a single experiment representative of 4 others.

CCL1-stimulated Ca^{2+} Mobilisation in RBLs is PLC-dependent

Calcium is an important and fundamental intracellular second messenger that relays information within cells to control processes as diverse as fertilization, proliferation, development, learning and memory, contraction and secretion (231). The release of calcium from internal stores held within the membrane systems of the ER is controlled by various channels of which the IP_3R has been studied most extensively (305). IP_3 is cleaved along with DAG from $PI(4,5)P_2$ by the receptor-activated enzyme PLC. Calcium-mobilising second messengers, such as IP_3, are generated when stimuli bind to cell surface receptors, diffuse into the cell to engage the IP_3Rs and release calcium from the ER. To determine whether the increase in intracellular calcium following ligation of CCR8 is a result of the enzymatic function of PLC, we used U73122, a pharmacological inhibitor of PLC. The concentration-dependent calcium response to CCL1 (Figure 19) was abrogated following a 30 minute pre-treatment of RBL-CCR8 cells with 1 μM of U73122. 1 μM of U73122 was used in this experiment because many studies have reported the IC_{50} of this inhibitor to be between 500 nM and 2.1 μM depending on the cell system involved (306-308). To verify that neither PTX nor the PLC inhibitor affect loading of cells with Fluo-4, the fluorescence of Fluo-4 loaded cells in the absence/presence of inhibitors was routinely analysed. Hence, fluorescence values were 10005 ± 91 and 9947 ± 90, 11218 ± 180 and 11020 ± 153 (mean ± SEM) in the absence and presence of PTX and PLC, respectively.

CCL1 stimulates the Phosphorylation of ERK1/2 in RBLs Transfected with CCR8

Activation of the MAPK pathway by other chemokines is well documented, and is usually represented by phosphorylation of ERK1/2. CCL1 induces a transient and time-dependent phosphorylation of ERK1/2 in RBL-CCR8 cells. The maximum intensity was consistently reached between 2 and 5 minutes post-stimulation. The phorbol ester 12-myristate 13-acetate, PMA, was used as a positive control for ERK1/2 activation. PMA activates PKC directly and links to the Ras/Raf/MEK/ERK1/2 pathway through the activation of the Ras guanyl nucleotide-releasing protein, Ras-GRP (309). The optimum concentration of CCL1 for maximal ERK1/2 phosphorylation was also investigated, and found to be between 3 and 10 nM (Figure 21).

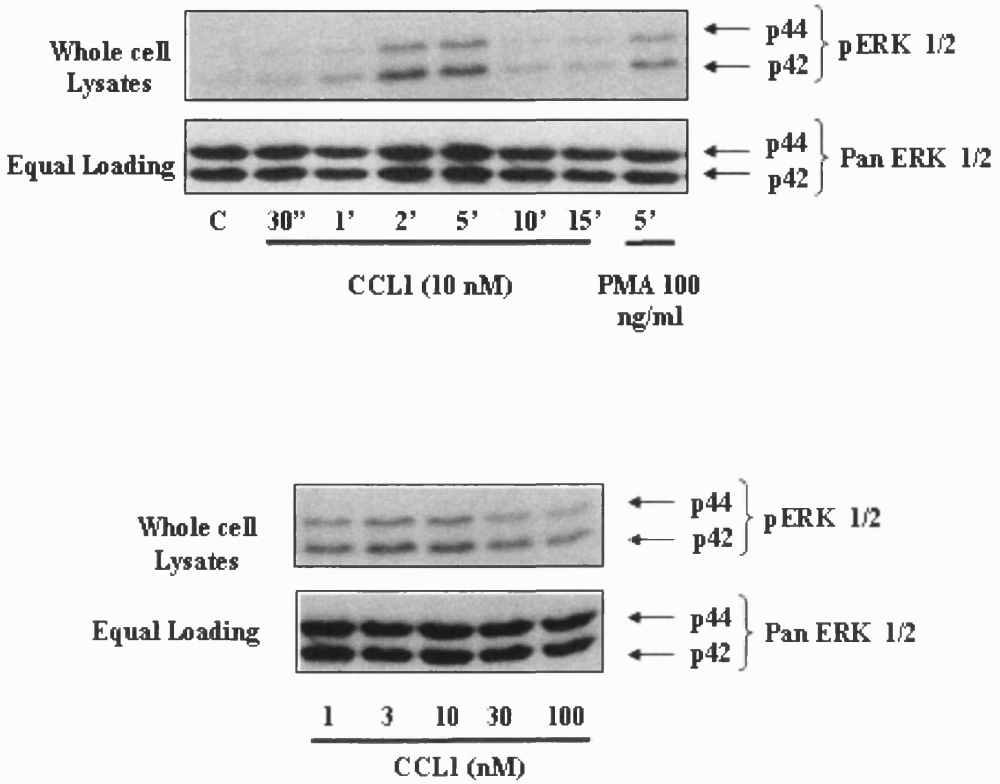

Figure 21. CCL1 induces the Phosphorylation of p42/44 ERK1/2 in Stably Expressing CCR8 transfected RBL cells.

RBL cells were aliquoted in 0.5 ml at $2x10^6$cells/ml and left unstimulated (C) or stimulated at 37°C with either the indicated concentration of CCL1 or 100 nM PMA. PMA was used as a positive control for ERK1/2 activation. The equivalent to $2x10^5$ cells of each sample was separated by SDS-PAGE. The non-stimulated control corresponds to both upper and lower blots as these data are derived from one experiment. Immunoblotting with the p44/42 phosphospecific ERK1/2 antibody was used to detect phosphorylated ERK1/2 proteins. The immunoblot was stripped and reprobed with the pan-ERK antibody that recognises both phosphorylated and non-phosphorylated forms of ERK1/2 to allow parity of loading to be examined. Data are from a single experiment representative of 2 others.

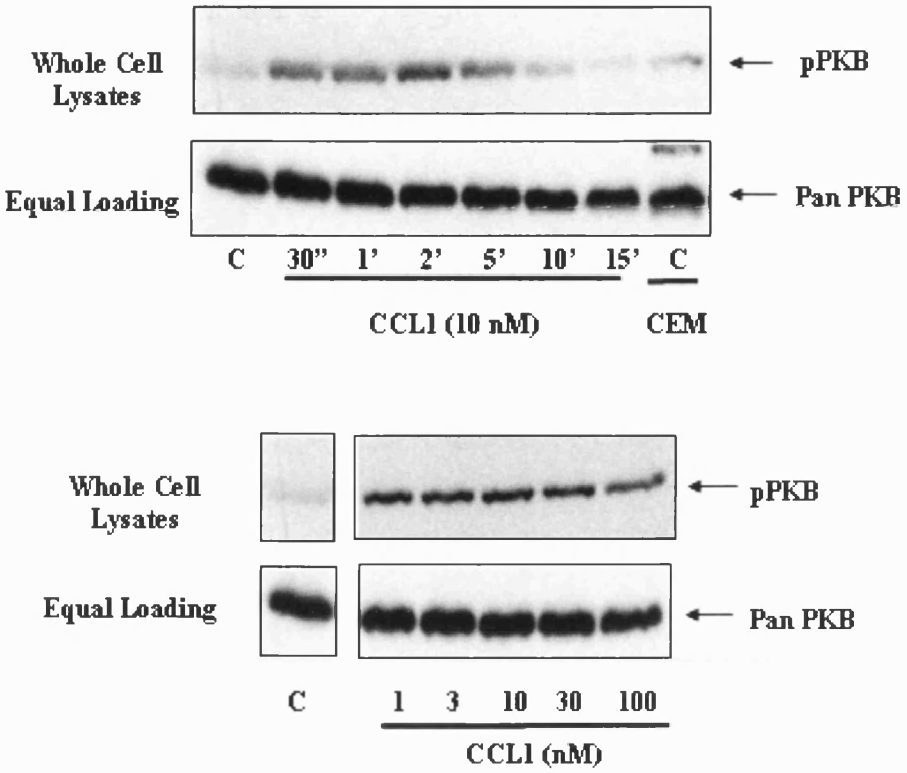

Figure 22. CCL1 induces Ser^{473} phosphorylation of PKB in CCR8-stably transfected RBL cells.

RBL cells were aliquoted in 0.5 ml at 2x10^{6}cells/ml and left unstimulated (C) or stimulated at 37°C with the indicated concentration of CCL1. CEM cells were used as a positive control for phosphorylated PKB due to their known high basal levels of Ser^{473}- phosphorylated PKB (293). The equivalent to 2x10^5 cells of each sample was separated by SDS-PAGE. The non-stimulated control corresponds to both upper and lower blots as these data are derived from one experiment. Immunoblotting with the phosphospecific Ser^{473} PKB antibody was used to detect phosphorylated PKB proteins. The immunoblot was stripped and reprobed with the pan-PKB antibody that recognises both phosphorylated and non-phosphorylated forms of PKB to allow parity of loading to be examined. Data are from a single experiment representative of 2 others.

CCL1/CCR8 Stimulates Activation of PI3K/PKB in RBL Cells

PI3K is an important enzyme involved in transducing signals from activated receptors (152). PKB is the major downstream effector of PI3K, its phosphorylation being dependent on D-3 phosphoinositide lipid formation. The phosphorylation of PKB is therefore used as an indirect marker of PI3K activation (299). A similar magnitude of PKB phosphorylation was observed at different concentrations of CCL1 between 1 and 100 nM. However, in previous studies using other chemokine receptors, a chemokine concentration of 10 nM has frequently been used (160) and consequently this concentration was chosen for this study. At 10 nM, CCL1 was shown to induce a transient but robust phosphorylation of PKB between 30 seconds and 5 minutes (Figure 22).

Use of Pharmacological Inhibitors to Abrogate CCR8-induced Phosphorylation of ERK1/2 and PKB

The following experiments were designed to identify the pathways that lead to the phosphorylation of ERK1/2 and PKB by utilising various pharmacological inhibitors. The use of these inhibitors facilitates the identification of molecules that are involved in certain cellular events. The majority of inhibitors are limited by their specificity, but if used at rational concentrations they can give a good indication of signalling pathways. The inhibitors chosen for this part of the study were the PI3K inhibitor LY294002, the MEK inhibitor PD98059 and the PKC inhibitor RO320432. The

mechanisms by which some of these inhibitors mediate their effects are unclear. LY294002 is reported to act on the ATP-binding site of PI3K, yet PD98059 does not compete for ATP binding or MAPK binding to MEK1 and most likely inhibits through an allosteric mechanism (310). Alessi *et al.* also found that PD98059 does not inhibit Raf-activated MEK1 but prevents the activation of MEK by Raf *in vitro* (311).

Using the inhibitors described above, experiments were carried out to determine the role of PI3K, MEK and PKC in the activation of both the ERK1/2-MAPK and PKB pathways. The effect of inhibitor concentration was investigated by using each inhibitor at concentrations of 3, 10 and 30 μM, based on published work (312-314). Unstimulated controls were incubated with the highest concentration of inhibitor (30 μM) to ensure they were not having any adverse effects. RBL-CCR8 cells were pre-incubated with each of the inhibitors for the same length of time as reported in other studies (312-314): LY294002 and RO320432 for 30 minutes; and PD98059 for 1 h. Cells were subsequently stimulated for 5 minutes with 10 nM of CCL1, as it had been previously demonstrated that CCL1 induces a maximum ERK1/2 phosphorylation at both 2 and 5 minutes (Figure 20), and a maximum PKB phosphorylation at 30 seconds, 1, 2 and 5 minutes (Figure 21).

As expected, a 5 minute stimulation of RBL-CCR8 cells with 10 nM of CCL1 resulted in a robust ERK1/2 phosphorylation above control levels (Figure 23). In the

presence of the PI3K inhibitor LY294002, the ERK1/2 phosphorylation was partially inhibited at all concentrations examined (3-30 μM). The MEK inhibitor PD98059 however, reduced the ERK1/2 phosphorylation in a concentration-dependent manner at 10, 20 and 30 μM. In contrast, the PKC inhibitor RO320432 had only a moderate effect on the phosphorylation of ERK1/2 at 30 μM (Figure 23).

A 5 minute stimulation of RBL-CCR8 cells with CCL1 resulted in the phosphorylation of PKB which in the presence of all concentrations of LY294002 was dramatically inhibited. In contrast, the MEK inhibitor PD98059 had a small effect on the PKB phosphorylation induced by CCL1. However, 10 and 30 μM RO320432 inhibited the phosphorylation of PKB whereas 3 μM had no effect (Figure 23).

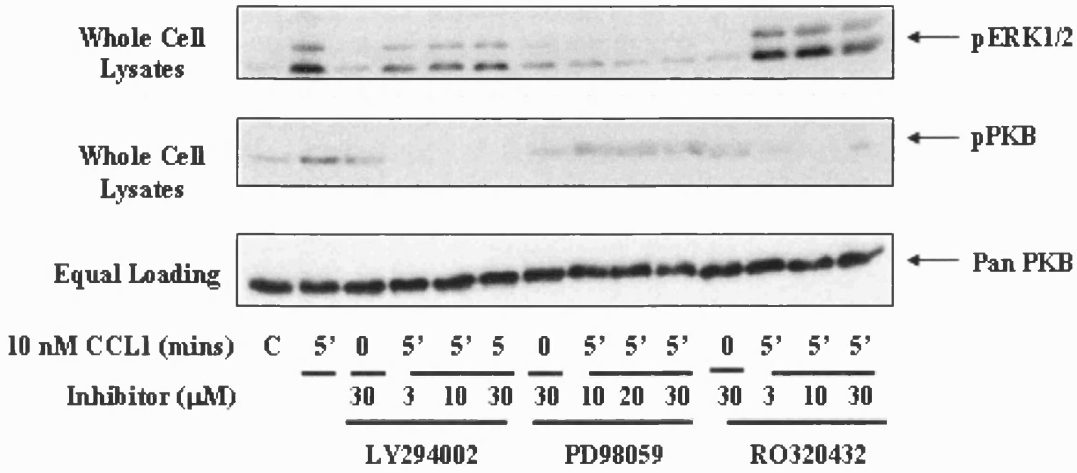

Figure 23. The Effect of PI3K, MEK and PKC Inhibitors on CCL1-mediated ERK1/2 and PKB Phosphorylation in RBL-CCR8 Cells

RBL cells were aliquoted in 0.5 ml at $2x10^6$cells/ml and pre-incubated with inhibitors for either 30 minutes in the case of LY294002 and RO320432 or 1 hr for PD98059. Cells were subsequently either left unstimulated (C) or stimulated at 37°C for 5 minutes with 10 nM CCL1. The equivalent to $2x10^5$ cells of each sample was separated by SDS-PAGE. Immunoblotting with either the p44/42 phosphospecific ERK1/2 or Ser^{473} PKB antibody were used to detect phosphorylated ERK1/2 and PKB. The immunoblot was stripped and reprobed with the pan-PKB antibody that recognises both phosphorylated and non-phosphorylated forms of PKB to allow parity of loading to be examined. Data are from a single experiment representative of 2 others.

4.3 CCR8 Signal Transduction in HUT-78 Cells

Several suspension cells, including *in vitro* differentiated human Th2 cells and Jurkat, HUT-78 and CEM T cell lines, were screened for CCL1-mediated responses. The leukaemic T cell line, HUT-78, were the only cells found to respond to CCL1 as demonstrated in both biochemical and functional responses. The use of HUT-78 cells would permit the investigation of CCL1-directed T cell migration and, since CCL1/CCR8 has been implicated in the recruitment of T cell subsets to the airways in a murine model of asthma (42,315,316), represents a more physiologically applicable system than that of transfected cells. Given that a transfected cell line may not contain the same expression profile of G proteins (among other molecules) as a cell line which has endogenous receptor expression, the first two experiments were designed to determine if HUT-78 cells responded to CCL1 stimulation in a similar way to that of RBL-CCR8 cells.

CCL1 Induces Ser^{473} Phosphorylation of PKB in HUT-78 Cells

HUT-78 cells stimulated with 10 nM CCL1 showed a rapid and transient, time-dependent phosphorylation of PKB (Figure 24). The T cell line CEMs were used as a positive control in this experiment for PKB phosphorylation because they have been shown to have constitutive PKB phosphorylation compared to peripheral blood lymphocytes, possibly due to the lack of PTEN (293). A similar phosphorylation of

PKB was observed in HUT-78 cells at different concentrations of CCL1 between 1 and 100 nM. However, in previous studies using other chemokine receptors, a chemokine concentration of 10 nM has frequently been used (287) and consequently this concentration was chosen for this study.

Use of Pharmacological Inhibitors to Elucidate the Mechanisms involved in CCL1-stimulated PKB Phosphorylation

The CCL1-stimulated signalling pathways in HUT-78 cells were examined using the pharmacological inhibitors described previously, in order to compare the sensitivity to these inhibitors relative to RBL-CCR8 cells. Only the PKB pathway was investigated, due to the surprising high basal levels of ERK1/2 found in this cell line. In this experiment, the HUT-78 cells were stimulated with 10 nM of CCL1 at their optimum time point for PKB Ser^{473} phosphorylation of 30 seconds (Figure 24).

A 30 second stimulation of HUT-78 cells with 10 nM of CCL1 resulted in the prominent phosphorylation of PKB which was inhibited by the PI3K inhibitor LY294002 in a concentration-dependent manner. In contrast, the MEK inhibitor PD98059 had a very limited effect on the PKB phosphorylation induced by CCL1. Only the highest concentration (30 μM) of PD98059 showed a modest inhibitory outcome. Moreover, the PKC inhibitor RO320432 also inhibited the phosphorylation of PKB in a concentration-dependent manner (Figure 25).

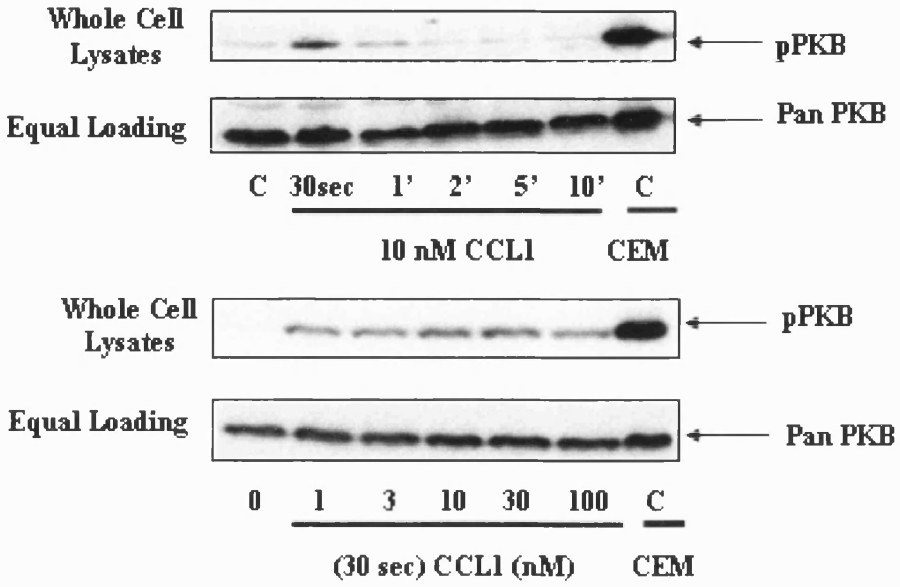

Figure 24. CCL1 Induces Ser^{473} Phosphorylation of PKB in HUT-78 Cells.

HUT-78 cells were aliquoted in 0.5 ml at 2x10^{6}cells/ml and left unstimulated (C) or stimulated at 37°C with the indicated concentration of CCL1. Unstimulated CEM cells were used as a positive control for phosphorylated PKB due to their known high basal levels (293). The equivalent to 2x10^5 cells of each sample was separated by SDS-PAGE. Immunoblotting with the phosphospecific Ser^{473} PKB antibody was used to detect phosphorylated PKB proteins. The immunoblot was stripped and reprobed with the pan-PKB antibody that recognises both phosphorylated and non-phosphorylated forms of PKB to allow parity of loading to be examined. Data are from a single experiment representative of 2 others.

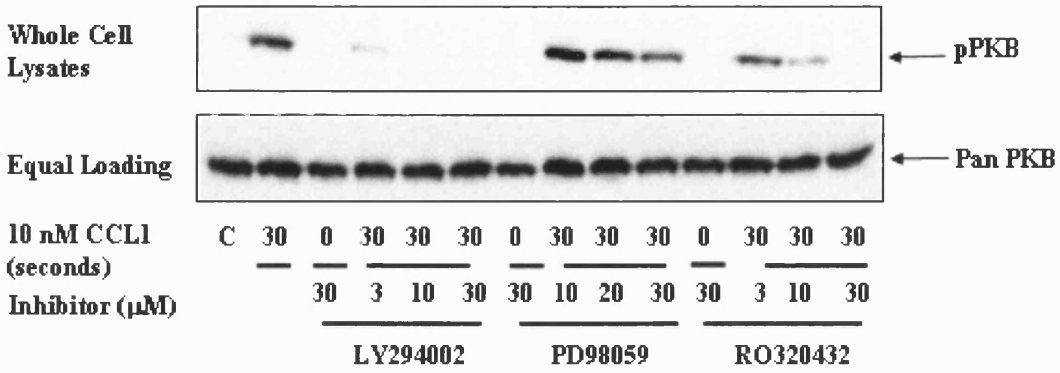

Figure 25. Effect of PI3K, PKC and MEK inhibitors on CCL1-mediated Ser^{473} PKB Phosphorylation in HUT-78 Cells

HUT-78 cells were aliquoted in 0.5 ml at $2x10^6$cells/ml and pre-incubated with inhibitors for either 30 minutes in the case of LY294002 and RO320432 or 1 hr for PD98059. Cells were subsequently either left unstimulated (C) or stimulated at 37°C for 30 seconds with 10 nM CCL1. The equivalent to $2x10^5$ cells of each sample was separated by SDS-PAGE. Immunoblotting with the Ser^{473} PKB antibody was used to detect phosphorylated PKB. The immunoblot was stripped and reprobed with the pan-PKB antibody that recognises both phosphorylated and non-phosphorylated forms of PKB to allow parity of loading to be examined. Data are from a single experiment representative of 2 others.

In vitro Chemotaxis of HUT-78 cells in Response to CCL1

Having established that activation of PI3K/PKB occurs following CCL1 stimulation of CCR8, this study sought to determine the functional significance of these pathways in the HUT-78 cell model. Chemotaxis, which is commonly used as a functional readout, is the process by which cells detect the direction and intensity of, and move toward, an extracellular chemoattractant gradient. This intriguing process plays a central role in development, immunity and tissue homeostasis (78-80). The migratory response of these cells to CCL1 was determined using disposable ChemoTx plates from Neuroprobe. These chemotaxis chambers enable the experiments to be carried out using small volumes of reagents. The CCL1-stimulated migration of HUT-78 cells exhibited classic bell-shaped concentration-dependent characteristics associated with chemokine responses in other systems (287,317) (Figure 26). Moreover, the chemotactic indices in response to all concentrations of CCL1 were greater than 4, and were found to be statistically significant. The chemotactic indices were calculated by dividing the mean fluorescence signal of migrated cells in chemokine-containing wells by the mean fluorescence signal of cells that migrated spontaneously toward media alone. Maximal responses were always observed with 1 nM CCL1 reaching a 9 fold chemotactic index over basal.

The Effect of PTX on CCL1-mediated Chemotaxis in HUT-78 Cells

Having previously demonstrated that CCL1-mediated Ca^{2+} mobilisation in RBL-CCR8 cells was dependent on $G_{\alpha i}$ (Figure 18), this study aimed to determine whether CCL1-directed cell migration of HUT-78 cells was also dependent on the $G_{\alpha i}$ subunit. HUT-78 cells were pre-treated with PTX (100 ng/ml) for 4 h and then subjected to a chemotaxis assay alongside untreated cells in response to CCL1 (1 nM). PTX treatment resulted in 86% inhibition of CCL1 stimulated chemotaxis (Figure 27). This inhibitory effect was found to be statistically significant. In order to ascertain whether PTX was affecting the viability of the HUT-78 cells, samples of cells, one treated with PTX and one untreated, were tested using trypan blue as a marker. Microscopic examination showed that there was no difference between the two samples, confirming that the PTX did not have a detrimental effect on cell viability.

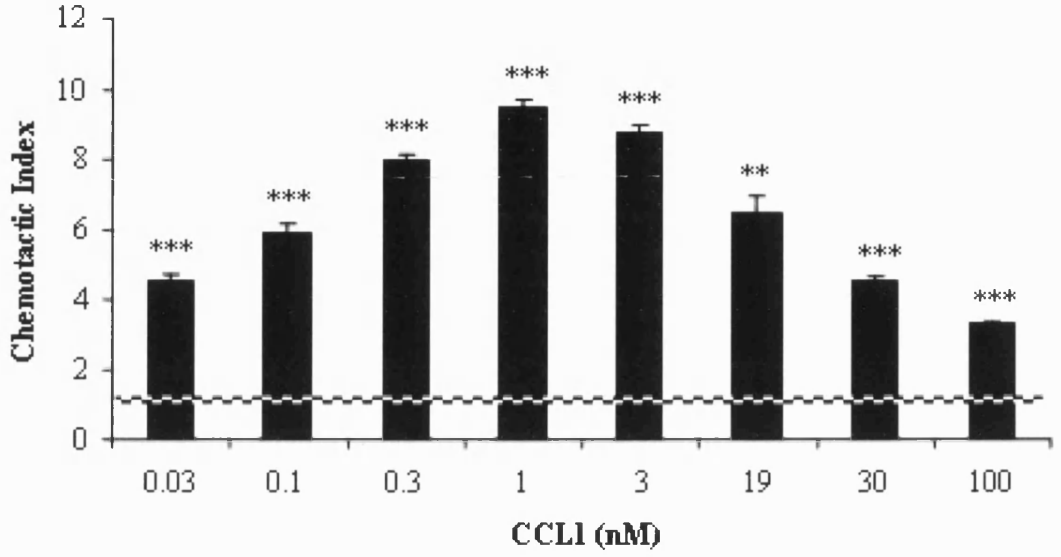

Figure 26. *In vitro* Chemotaxis of HUT-78 Cells in Response to CCL1

HUT-78 cells were loaded with Calcein-AM dye for 30 minutes. The cells were then washed and re-suspended at $2.8x10^6$/ml in 0.1% BSA RPMI. 29 µl of either CCL1 (between 0.03 and 100 nM) or buffer alone was added to the base of a disposable chemotaxis chamber. The 5 µm filter was attached as described in *Material and Methods* and 25 µl of cells added to the top. The chemotaxis plate was incubated for 90 minutes at 37°C, 5% CO_2, then washed of residual cells and read on a fluorescent plate reader at 485 nm excitation and 538 nm emissions using the bottom reading mode. Data were analysed by Student's *t* test to determine the significance of CCL1 concentration compared to cells migrating randomly in response to media alone (**, $p < 0.0002$; ***, $p < 0.00005$). The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels. Data are from a single experiment representative of 3 others.

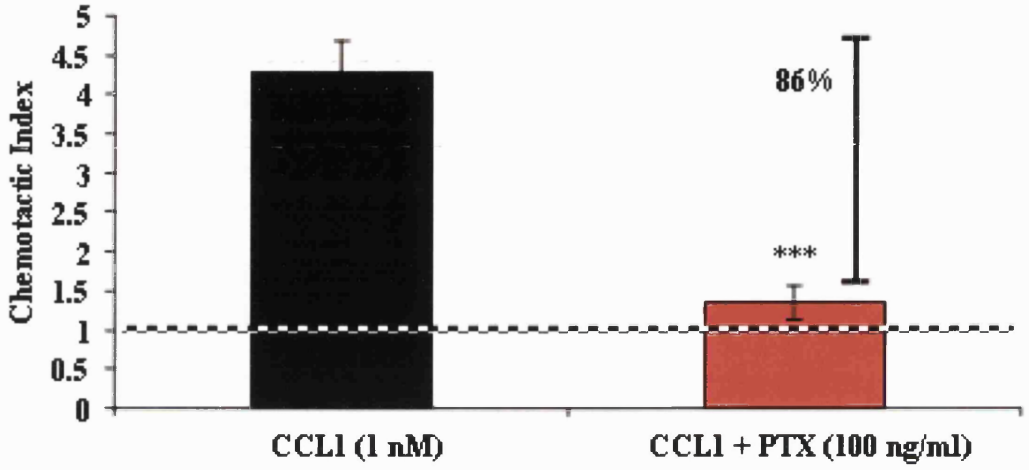

Figure 27. CCL1-mediated Chemotaxis of HUT-78 Cells is $G_{\alpha i}$-dependent

HUT-78 cells were treated with PTX for 4 h prior to loading with Calcein-AM for 30 minutes. The cells were then washed and re-suspended at $2.8x10^6$/ml in 0.1% BSA RPMI. 29 µl of either CCL1 (1 nM) or buffer alone was added to the base of a disposable chemotaxis chamber. The 5 µm filter was attached as described in *Material and Methods* and 25 µl of cells added to the top. The chemotaxis plate was incubated for 90 minutes at 37°C, 5% CO_2, then washed of residual cells and read on a fluorescent plate reader at 485 nm excitation and 538 nm emissions using the bottom reading mode. Data were analysed by Student's *t* test to compare responses in the presence and absence of PTX (***, $p < 0.00005$). The percentage inhibition of migrating cells as a result of PTX treatment is indicated on the graph. The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels. Data are from a single experiment representative of 3 others.

The Importance of PI3K in CCL1-mediated Chemotaxis of HUT-78 Cells

Until recently, PI3K was believed to be crucial for chemokine-mediated chemotaxis (160). However, Smit *et al* (317) recently reported the PI3K-independent chemotaxis of human T cells in response to CXCR3 ligands. Similarly, chemotaxis of CCR4-expressing CEM cells in response to CCL22/MDC and CCL17/TARC have been shown to be insensitive to the PI3K inhibitors LY294002 and Wortmannin at 30 μM and 300 nM respectively (*Cronshaw and Ward, unpublished data*).

This section aims to examine whether PI3K is required for the movement of HUT-78 cells in response to CCL1. HUT-78 cells were pre-treated with LY294002 at 3, 10 and 30 μM and subsequently incubated in a standard chemotaxis assay with 1 nM CCL1, the concentration found to elicit maximal chemotactic response. In this experiment CCL1 alone induced a chemotactic index of greater than 2.5 in HUT-78 cells, whilst pre-treatment with the PI3K inhibitor LY294002 abrogated chemotaxis in a concentration-dependent manner (Figure 28).

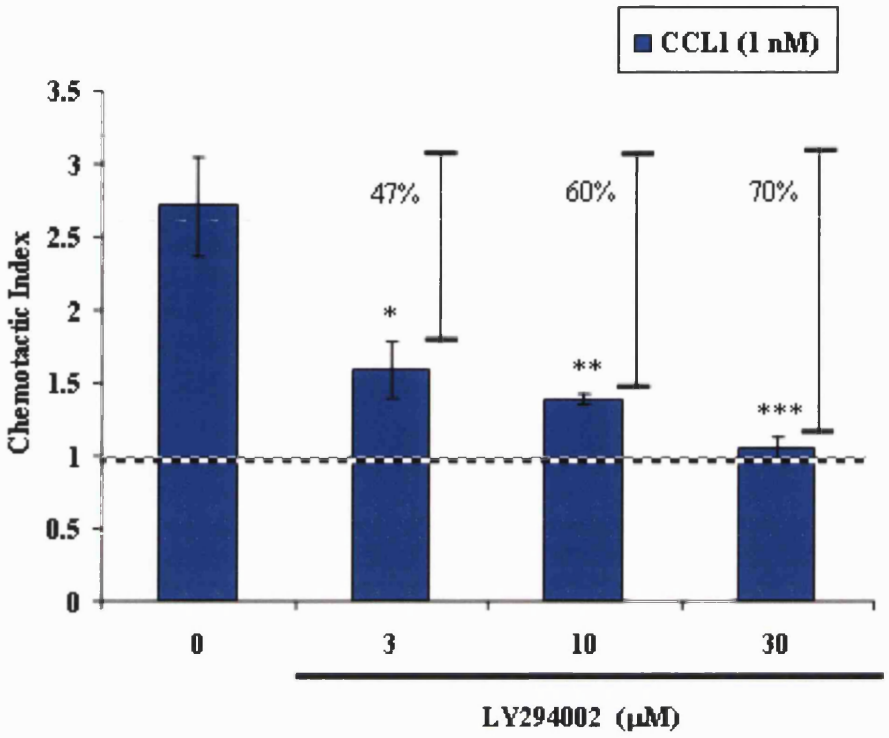

Figure 28. CCL1-mediated Chemotaxis of HUT-78 Cells is PI3K-dependent

HUT-78 cells were pre-loaded with Calcein-AM for 30 minutes, washed and re-suspended at $2.8x10^6$/ml in 0.1% BSA RPMI. LY294002, at the indicated concentrations, was incubated with cells for 30 minutes prior to loading with Calcein. 29 μl of either CCL1 (1 nM) or buffer alone was added to the base of a disposable chemotaxis chamber. The 5 μm filter was attached as described in *Material and Methods* and 25 μl of cells added to the top. The chemotaxis plate was incubated for 90 minutes at 37°C, 5% CO_2, then washed of residual cells and read on a fluorescent plate reader at 485 nm excitation and 538 nm emissions using the bottom reading mode. Data were analysed by Student's *t* test to compare responses in the presence and absence of LY294002 (*, $p < 0.002$; **, $p < 0.00005$; ***, $p < 0.000005$). The percentage inhibition of migrating cells as a result of LY294002 treatment is indicated on the graph. The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels. Data are from a single experiment representative of 3 others.

Use of Inhibitors to Elucidate the Role of PLC-dependent Signalling Pathway in Chemotaxis

To establish which pathways are involved in CCL1-mediated chemotaxis of HUT-78 cells, a selection of inhibitors were used to examine the role of PLC, IP_3-mediated intracellular Ca^{2+} release and PKC. These were the PLC inhibitor U73122, U73343 (a weak analogue of the PLC inhibitor) (318) and the PKC inhibitor RO320432. A compound termed 2APB, has been reported to inhibit IP_3-mediated calcium release without blocking the binding of IP_3 to its receptor (319) and this reagent was also used in this study. Shown in Figure 29 is a representative result of chemotaxis assays performed on the HUT-78 cells. Cells were pre-treated with the above inhibitors for 30 minutes prior to the assay. CCL1 (1 nM) stimulated the chemotaxis of HUT-78 cells alone with a chemotactic index of greater than 4. Treatment with the PLC inhibitor U73122 (3 μM) resulted in a 70% inhibition of migration of HUT-78 cells; however the weak analogue of U73122, U73343 failed to block the chemotaxis of cells (5% inhibition) in response to CCL1. To identify whether the source of calcium that regulates chemotaxis is dependent on IP_3, cells were pre-incubated with the inhibitor 2APB (50 and 75 μM). At 50 μM, 19% inhibition was found; whilst at 75 μM inhibition was 45%. Finally the PKC inhibitor, RO320432 (10 μM) was found to inhibit the chemotactic response of HUT-78 cells by 60% whilst the MEK inhibitor PD98059 was only able to reduce the CCL1-mediated migration of HUT-78 cells by 18%.

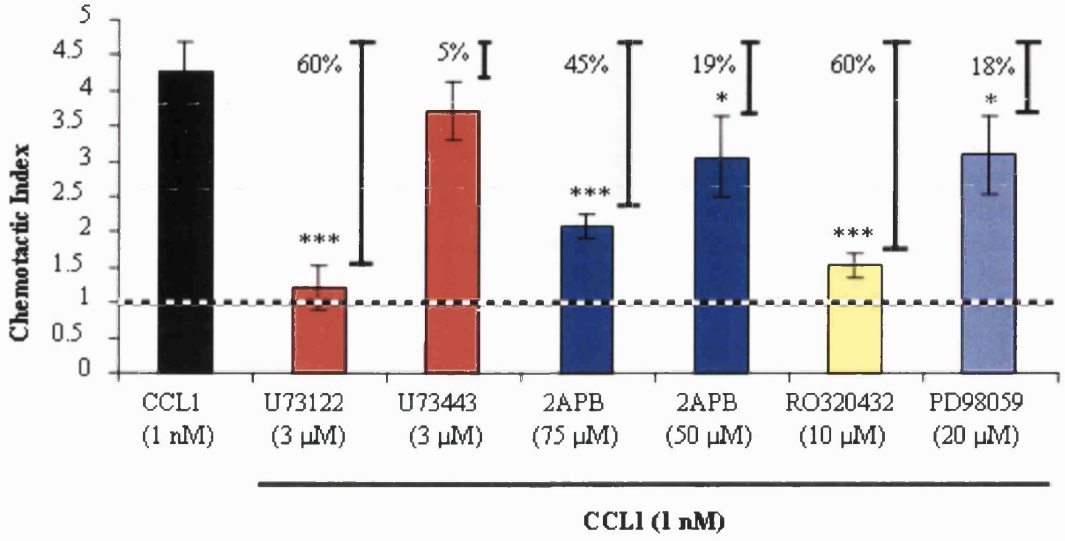

Figure 29. Effect of Pharmacological Tools on CCL1-mediated Chemotaxis of HUT-78 Cells

HUT-78 cells were pre-loaded with Calcein-AM for 30 minutes, washed and re-suspended at $2.8x10^6$/ml in 0.1% BSA RPMI. Inhibitors at the indicated concentrations were incubated with cells for 30 minutes prior to loading with Calcein. 29 µl of either CCL1 (1 nM) or buffer alone was added to the base of a disposable chemotaxis chamber. The 5 µm filter was attached as described in *Material and Methods* and 25 µl of cells added to the top. The chemotaxis plate was incubated for 90 minutes at 37°C, 5% CO_2, then washed of residual cells and read on a fluorescent plate reader at 485 nm excitation and 538 nm emissions using the bottom reading mode. Data were analysed by Student's *t* test with Bonferroni's correction for multiple comparisons to compare responses in the presence and absence of inhibitors (*, $p < 0.00048$; ***, $p < 0.00000006$). The percentage inhibition of migrating cells as a result of inhibitor treatment is indicated on the graph. The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels. Data are from a single experiment representative of 3 others.

Summary of Findings

- CCR8 was confirmed to be expressed on RBL-CCR8 cells.

- CCL1 mediates a $G_{\alpha i}$- and PLC-dependent calcium mobilisation in RBL-CCR8 cells.

- CCL1 induces a time- and concentration-dependent activation of the MAPK pathway in RBL-CCR8 cells.

- Activation of the MAPK pathway is dependent on MEK, partially dependent on PI3K and independent of PKC in RBL-CCR8 cells.

- CCL1 induced a time-dependent phosphorylation of PKB in both RBL-CCR8 and HUT-78 cells.

- Activation of PKB is dependent on PI3K, partially dependent on PKC but independent of MEK in both RBL-CCR8 and HUT-78 cells.

- CCL1 stimulates the chemotaxis of HUT-78 cells in a concentration-dependent manner with bell-shaped characteristics.

- The chemotactic response of HUT-78 cells to CCL1 is dependent on $G_{\alpha i}$, PI3K, PLC and PKC and partially dependent on IP_3.

4.4 Discussion

Detecting the Expression of CCR8 on Cell Membranes

Detecting the expression of human CCR8 on the surface of cells used in this study was restricted by the availability of a reliable antibody for this receptor; a few have been marketed but none to date have been successful *(Brown Z. Novartis Pharmaceuticals, personal communication)*. An alternative method was used to detect the expression of CCR8 through the binding of a biotinylated CCL1 and avidin-fluorescein. However, it seemed that this method was only useful for the detection of high levels of receptor expression, because CCR8 could not be detected on HUT-78 cells or T-lymphoblasts even though biochemical responses through CCR8 were prominent in these cells. Alternatively, it is possible that an additional un-characterised receptor for CCL1 exists.

CCL1-mediated Calcium Mobilisation is Abrogated by PTX and the PLC Inhibitor, U73122

The results in this study reveal that CCL1 mediates a PLC-activated calcium flux in RBL-CCR8 cells that is entirely PTX-sensitive. Whilst PTX does indicate that a response is dependent on Gαi-coupling, it does not distinguish between the involvement of either the αi or βγ subunits mediating activation of various pathways. Indeed, there is also no evidence for $G_{\alpha i}$ binding to PLCβ, and it is well established

that Ca^{2+} signalling by many $G_{\alpha i}$ -coupled receptors is via $G_{\beta\gamma}$-mediated activation of PLCβ (320-322). Each of the four identified PLCβ isoforms can be stimulated by $G_{\beta\gamma}$ subunits (323,324) with marked differences in their sensitivities (323). The majority of chemokine-mediated calcium responses are PLCβ-dependent, however in contrast to the results presented in this thesis, not all are reliant on the coupling of the $G_{\alpha i}$ subunit to the receptor. One study reported that chemokine-elicited intracellular calcium responses in freshly isolated and cultured cerebellar Purkinje and granule neurons were PTX-insensitive (325).

CCR8-mediated Chemotaxis of HUT-78 Cells

The crucial role of G proteins in chemotaxis of lymphocytes is well known. It is commonly accepted that all chemokine receptors couple to $G_{\alpha i}$, although some chemokine receptors can also couple to $G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha 14}$, and $G_{\alpha 16}$ proteins (326,327). Pre-treatment of HUT-78 cells with PTX completely blocks CCR8-mediated chemotaxis, suggesting that this response is entirely dependent on Gαi-coupling.

Activation of PI3K and the accumulation of its lipid product, $PI(3,4,5)P_3$, is not only essential for the establishment of the leading edge of a migrating cell but is also required for cell polarization and directional movement in simple organisms such as *Dictyostelium discoideum*, as well as in more complex mammalian cells (328-330).

Furthermore, it has been demonstrated that protein kinase B (PKB), a major effector of the PI3K-dependent signaling cascade that interacts with $PI(3,4,5)P_3$ and $PI(3,4)P_2$ via its PH domain, colocalizes with these lipids and filamentous actin at the leading edge (331). The data from our study demonstrate for the first time that CCL1-directed cell migration is dependent on PI3K in HUT-78 cells. Despite these exciting developments, it is unclear which PI3K isoforms are involved in the accumulation of $PI(3,4,5)P_3$ and $PI(3,4)P_2$ in response to chemokines. The G protein-sensitive PI3Kγ is an obvious candidate for the accumulation of $PI(3,4,5)P_3$ in response to chemokine stimulation of G protein-coupled chemokine receptors, as it is activated by Gβγ following ligation of GPCRs (332). However, there is evidence that GPCRs are able to activate class IA PI3Ks. The class IA, p110δ has been shown to be important for both fMLP-induced neutrophil migtration and in controlling the directionality of EGF-driven breast cancer cell migration (333,334). In addition, recent work suggests that the optimal chemotactic response of leukaemic T cells to CXCL12/SDF-1 requires activation of both class IA and IB PI3Ks (287).

The PI3K isoforms that are responsible for CCL1-directed, PI3K-dependent migration of HUT-78 cells have not been investigated in this thesis. However, since HUT-78s express all class IA and IB PI3K isoforms, in addition to the class II isoforms, C2α and C2β, then it is plausible that p110γ and p110δ may have roles to play in CCL1-directed cell migration of HUT-78 cells. To date, research on class II PI3Ks is limited, although activation of PI3K-C2α by the chemokine CCL2/MCP-1

has been shown in THP-1 cells (197). LY294002 is reported to inhibit all PI3K isoforms with an IC_{50} in the 1-50 μM range (335). In our hands, low concentrations of LY294002 (3 μM) can only inhibit class I PI3Ks, whereas class II PI3Ks begin to be inhibited by LY294002 at approximately 30 μM *(Kouroumalis and Ward, unpublished data).* Given that CCL1-directed migration of HUT-78 cells was inhibited by the PI3K inhibitor, LY294002, at 3 μM, it is more likely that class I PI3Ks are involved.

The activity of PI3K and thus accumulation of $PI(3,4,5)P_3$ at the sides and back of the cell are restricted by the lipid phosphatase PTEN. $PI(4,5)P_2$ has been reported to be responsible for the localisation of PTEN to the plasma membrane at the back of the cell. Such models imply that $PI(4,5)P_2$ is depleted from the leading edge in order to prevent the recruitment of PTEN, this is believed to occur not from its phosphorylation by PI3K, but from the action of PLC induced by chemokine signalling (328,329).

The Role of Calcium and PKC in Cell Migration

The presence of the PLC-signaling pathways in T cells and the ability of chemokines to activate this system were previously demonstrated for CXCL8/CXCR2 (336). Moreover, PLCβ activation of heterologously expressed chemokine receptors was shown for CCR1 and 2 (326). The role of PLC as a determinant of chemokine-

mediated chemotaxis has recently been demonstrated in two diverse systems by Smit *et al* and Soriano *et al.* The PLC inhibitor, U73122 (10 μM) was shown to abolish both CXCL11/I-TAC-directed cell migration of activated human T lymphocytes (317) and CXCL12/SDF-1-directed cell migration of MOLT4 cells *in vitro* (290). Similarly, this study reveals that migration of HUT-78 cells in response to CCL1 is dependent on PLC, as demonstrated using 3 μM of U73122. Together with the data reported in this section, the PLC pathway appears to play a role in chemokine-mediated T cell migration.

The Role of Calcium and PKC in CCL1-directed Cell Migration of HUT-78 Cells

The hydrolysis of $PI(4,5)P_2$ by PLC, generating the second messengers IP_3 and DAG is well documented. The binding of IP_3 to the IP_3R present on the surface of ER/SR induces the release of calcium into the cytoplasm. Changes in cytosolic Ca^{2+} concentrations evoke a wide range of cellular responses and intracellular calcium-binding proteins are the key molecules to transduce Ca^{2+} signalling via enzymatic reactions or modulation of protein/protein interactions. These include the calcium/phospholipid binding proteins like PKCα/β which are activated by Ca^{2+} and DAG and 'EF hand' proteins like calmodulin (CaM) and S100 proteins, which are considered to exert Ca^{2+}-dependent actions in the nucleus or the cytoplasm. The binding of Ca^{2+}/CaM to the myosin light-chain kinase (MLCK) modulates the

functional association between actin and myosin II and thus provides the force necessary for lamellipod extension and uropod retraction (231).

Chemokines have been reported to mobilise Ca^{2+}, although the requirement for calcium for chemokine-mediated cell migration remains ambiguous. Immediately after the initial Ca^{2+} release from the central store, in both the giant newt eosinophil (337) and *Dictyostelium* (338), the Ca^{2+} concentration falls most rapidly in the part of the cell nearest the chemoattractant source so that a back-to-front Ca^{2+} concentration gradient forms. This small gradient, from 250 nM in the uropod to ~100 nM in the lamellipod, could be responsible for the differing functions of Ca^{2+}-dependent processes in the newly polarised cell. In migrating fibroblasts, a similar gradient in the distribution of Ca^{2+}/CaM has also been reported (339). The Ca^{2+} gradient could be because of the relative distributions of IP_3 and DAG in the newly stimulated cell (340). Thus giant newt eosinophils and *Dictyostelium* amoebae become polarized, not only in the distribution of major components of the cytoplasm such as nucleus and granules, but in the gradients of cytosolic free Ca^{2+} concentration and DAG distribution (337). A Ca^{2+} gradient of this type has been difficult to demonstrate or is apparently absent in smaller cell types, such as human neutrophils (341), and these cells are able to undergo actin polymerization (342) and effective chemotaxis (343) in the absence of, or incidental to (344) Ca^{2+} signaling, which seems to be necessary for eosinophil chemotaxis (345). These observations may suggest that a Ca^{2+} signal is not essential for the generation of polarity. In the giant newt eosinophil, establishment of

a polarized morphology may depend on elongation of the cell along an innate axis, determined by the arrangement of microtubules, rather than the chemotactic gradient (346).

This study utilised the pharmacological inhibitor 2APB which at high concentrations is described as an inhibitor of IP_3-mediated calcium release. CCL1-mediated chemotaxis of HUT-78 cells was shown to be diminished in the presence of 50-75 μM 2APB, implicating a role for Ca^{2+} in CCL1-mediated T cell migration. In contrast, chemotaxis of CCR4-expressing CEM cells in response to CCL22/MDC have been shown to be insensitive to 2APB (75 μM) (*Cronshaw and Ward, unpublished data*). Taken together, these data would suggest that the dependency on Ca^{2+} is cell-type, as well as chemokine receptor specific.

The second product of PLC-mediated hydrolysis of $PI(4,5)P_2$ is DAG, which is known to activate PKC. The family of PKC consists of at least 10 isoforms, and each of them exhibits a unique pattern of tissue distribution, subcellular translocation, and function (347). The PKC isoforms can be divided into three subfamilies: classical PKCs (cPKCs), such as α, βI, βII, γ require both Ca^{2+} and DAG for activation; novel PKCs (nPKCs), such as δ, ε, η,θ, are DAG-dependent but Ca^{2+}-independent; and atypical PKCs, such as ζ and λ require neither Ca^{2+} or DAG. PKC has been studied in many different cell types and has been shown to be important in chemotaxis in

response to different stimuli (348-351). An impressive study which used antisense oligodeoxyribonucleotides (ODN) to specifically inhibit PKCβ demonstrated that PKCβ is essential for MCP-1 stimulated chemotaxis of human monocytes (352). PKCζ has been shown to be essential in mediating both IL-8 and fMLP-induced neutrophil chemotaxis and regulating the polarity of astrocytes during wound healing process (353,354). This study relied on the use of the pharmacological inhibitor, RO320432 to block PKC, which is reported to be 10-fold more selective for PKCα and 4 fold more selective for PKCβI over PKCδ (355). CCL1-mediated cell migration of HUT-78 cells was abrogated in the presence of 10 μM RO320432. Since the migration of HUT-78 cells in response to CCL1 is Ca^{2+}- and PKC-dependent, then it is likely that one of classical PKC isoforms is required for CCL1-mediated migration of HUT-78 cells.

Chemokines Induce a PKC-dependent Phosphorylation of PKB

The dependency on PKC in the activation of the PKB pathway is complex due to the differing functions of the many PKC isoforms. Only a small number of isoforms have been investigated with respect to PKB, so the precise role of each isoform remains ambiguous. PKCs (356) (and more specifically PKCζ (357)) have been described to function as negative regulators of PKB activity where pre-incubation of cells with inhibitors led to enhanced PKB phosphorylation. In contrast, the vascular endothelial

growth factor (VEGF) is reported to induce PKC-dependent PKB activation (358) which implies PKC resides upstream of PKB in this system. Similarly, PKB activation by a synthetic chemoattractant peptide (WKYMVm) was found to be dependent not only on PI3K but also on the PKC pathway (359). The data presented in this study is in agreement with the notion that PKC lies upstream of PKB, as CCL1-mediated Ser^{473} phosphorylation of PKB was markedly inhibited by the PKC inhibitor, RO320432 in both RBL-CCR8 and HUT-78 cells.

This Study Provides the First Evidence that CCR8 is Coupled to the PI3K/PKB Signalling Pathway

All stimuli in lymphocytes that induce the production of D-3 phosphoinositides including antigen receptors, cytokines and chemokines are now known to activate PKB. Thus it does not come as a surprise that CCL1 stimulates the phosphorylation of PKB in both CCR8-expressing RBL and HUT-78 cells in this study. The magnitude and duration of the PKB response induced by antigen receptors and IL-2 are similar and PKB activation by these stimuli is maintained for several hours (360). In contrast, chemokine activation of PKB in lymphocytes is very transient and only sustained for a few minutes (361). These kinetics are evident in this study where CCL1 stimulates the phosphorylation of Ser^{473} PKB as early as 30 seconds in both RBL and HUT-78 cells, with a diminished Ser^{473} phosphorylation by 1 minute in HUT-78 cells and 10 minutes in RBL-CCR8 cells. It is quite possible that different

lymphocyte subpopulations will differ in their kinetic rate of PKB inactivation because they differ in the regulation of protein phosphatases, such as PP2A, that remove the activating phosphates on PKB. In addition, given that (i) the expression of dominant-negative constructs of class IA and class IB PI3Ks leads to partial inhibition of CXCL12-stimulated PKB (287) and that (ii) activation of PKB by CXCL12 is prevented by PI3K inhibitors (195), it is not surprising that CCL1-mediated phosphorylation of PKB is also abrogated in the presence of the PI3K inhibitor LY294002.

Cross-talk between MAPK and PI3K/PKB Pathways

The serine/threonine kinase PAK has emerged as a molecule that can link PI3K/PKB to the ERK MAPK pathway. Receptor-mediated activation of Raf, the upstream MAP kinase kinase kinase in the ERK cascade, is Ras-dependent and involves recruitment of the kinase to the plasma membrane. Several groups have reported synergism between Rho GTPases and this pathway. It is widely reported that overexpression of a Rho GTPase cannot in itself lead to ERK activation, however, dominant-negative Rac can block Ras-dependent ERK activation in human kidney fibroblast 293 cells (362). Furthermore, Rac or Cdc42 can synergize with Raf to promote ERK activation and this synergy has been reported to occur at the level of the MAP kinase kinase, MEK1 (363). It turns out that PAK, which is a Cdc42 and Rac target, can phosphorylate MEK1 on Ser298 in a region that mediates the interaction of MEK1

with Raf. More recent work has introduced more complexity to this cross-talk mechanism, since PAK can also phosphorylate Raf on Ser338, and this appears to be essential for integrin-mediated activation of ERK in COS7 cells (364). Activation of PAK in these cells is dependent upon PI3K-dependent activation of Rac (365), though in Rat-1 cells, PAK activation occurs through a PI3K-mediated activation of another serine/threonine kinase, PKB and independently of Rac (366). Data from our study showed that stimulation of RBL and HUT-78 cells with CCL1 resulted in a MEK-independent phosphorylation of PKB, implying that the MAPK pathway is not required for the activation of PKB in this model system. Similarly, IL-3 and GM-CSF stimulated MC/9 cells were shown to induce a MEK-independent phosphorylation of PKB (367). Taken together, these published studies suggest that there is a link between the PI3K/PKB and Ras/Raf/MEK/ERK pathway, although PKB appears to lie upstream and converges with MEK through activation of the serine/threonine kinase PAK.

Robust phosphorylation of ERK1/2 by CCR8 in transfected RBL cells

In accordance with the role of the MAPK pathway in regulating cell growth and differentiation, activation of this pathway has been shown to mediate anti-apoptotic activity of CCL1 and vMIP-I in the BW5147 lymphoma (368), however, the molecular machinery that links CCR8 signalling to ERK1/2 activation in T cells has not been fully characterised. This study shows for the first time that CCL1-mediated

ERK1/2 activation follows a similar pathway to that seen by other chemokines. Multiple studies have implicated MEK and PKC as positive regulators of MAPK activation in response to various mitogenic stimuli (369-371). Of particular resemblance to the current investigation, activation of PKCε, MEK and ERK1/2 were recently shown to participate in the regulation of MCP-1 expression on vascular endothelial cells by shear stress. Pharmacological inhibitors of PKCβ/ε (Calphostin C) and MEK (PD98059) were demonstrated to inhibit MCP-1 mediated ERK1/2 activation in addition to repressing MCP-1 induction (370). The specific inhibitor of PKCβ (Go96976) however, had no effect on either ERK1/2 activation or MCP-1 induction, further supporting a role for PKCε in this system. The ERK1/2 pathway has, more specifically, been attributed to the regulation of thymocyte development. Werlen *et al* showed that a motif in the αβ T-cell receptor (TCR) controls positive thymocyte selection by modulating ERK1/2 activity (372). Having demonstrated in this study that stimulation of CCR8-expressing RBL cells with CCL1 leads to activation of the ERK1/2 MAPK pathway and CCR8 is preferentially expressed in the thymus (31), one might speculate that CCR8 may play a role in the regulation of thymocyte development through activation of the ERK1/2 MAPK cascade. Similarly, because the ERK1/2 pathway also contributes to T lymphocyte immune activation (373), ERK1/2 activation is likely involved in the regulation of these processes by CCL1.

Identification of the pathways that lead to ERK1/2 phosphorylation

Protein phosphorylation is an important molecular mechanism by which extracellular signals produce biological responses in cells; therefore this study examined the ability of CCL1 to activate protein kinase pathways in RBL-CCR8 cells. A recent study reports that CCR8-dependent activation of the Ras/MAPK pathway mediates anti-apoptotic activity of CCL1 and vMIP-I, yet the mechanisms by which this occurs has not been investigated. GPCRs are known to activate ERK1/2 by numerous mechanisms which remain the subject of intense investigation (252), although the pathways that lead to chemokine-mediated ERK1/2 phosphorylation appear to have some similarities. To dissect the cascade of events leading to MAPK activation upon CCR8 receptor stimulation, several inhibitors of signal transduction proteins were used in RBL cells transfected with CCR8 receptors. This study shows that ERK1/2 activation by CCL1 is dependent on MEK, partially dependent on PI3K and independent of PKC. In comparison to the well-characterised CXCR4/CXCL12 receptor chemokine system, the signalling pathways that lead to ERK1/2 phosphorylation following ligation of CCR8 with CCL1 are surprisingly analogous. The only controversial issue with CXCL12-mediated ERK1/2 phosphorylation is the dependency on PI3K. Some studies have reported ERK1/2 activation to be dependent on PI3K (195,374) yet others believe that it is independent (287).

The importance of PI3K within the ERK1/2 MAPK pathway is far less understood. Previously, many groups have shown that chemokine-stimulated ERK1/2 activation in various leukocytes is inhibited by PI3K inhibitors, suggesting a PI3K-dependent activation of the MAPK pathway (194,195,375). IL-8, for example, was reported to activate the Ras/Raf/ERK1/2 pathway in human neutrophils in a PI3K-dependent manner. However in other studies chemokine-mediated activation of ERK1/2 has proven to be refractory to PI3K inhibition (287,376). Jurkat T cell clones stably expressing dominant-negative constructs of class IA and IB PI3K were shown to have no effect on CXCL12-induced phosphorylation of the MAPK ERK1/2 (287). Similarly, our study demonstrates that CCL1-mediated ERK1/2 phosphorylation is independent of PI3K in CCR8-expressing RBL cells. These discrepancies may be explained in terms of different chemokines coupling to MAPK activation via distinct signalling pathways.

The Role of ERK/MAPK in Chemokine-mediated Cell Migration

While MAPK signalling has been associated with the regulation of gene transcriptional events, evidence now suggests that MAPK can promote cell migration on the extracellular matrix in a transcription-independent manner. MAPK has been reported to directly phosphorylate MLCK, thereby increasing its ability to phosphorylate MLC, which promotes cytoskeletal contraction necessary for cell movement (377,378). Inhibition of ERK activation by the use of ERK inhibitors such

as PD98059 abrogates CXCL12/SDF-1, MIP-3α and eotaxin-induced actin polymerisation and/or migration of T cells or eosinophils (195,196). However, chemotaxis of neutrophils in response to the chemokine IL-8 (194) or non-chemokine chemoattractants such as fMLP and C5a is not blocked by the MEK inhibitor PD98059 (379-381). Similarly, the results presented in this study revealed that CCL1-mediated cell migration of HUT-78 cells was also insensitive to PD98059. The different sensitivity to MEK inhibition of cell migration in response to chemokines and other chemoattractants, suggests that there are multiple pathways leading to leukocyte chemotaxis and highlights the fact that even though different chemokine receptors can share biochemical signalling pathways, there is sometimes a degree of redundancy with respect to the importance of those pathways in evoking a chemotactic response.

4.5 Concluding Remarks

This section has identified some of the signalling pathways that are mediated by the CCL1/CCR8 chemokine/chemokine receptor system. Firstly, this study has shown that CCL1 mediates a $G_{\alpha i}$- and PLC-dependent calcium mobilisation which could lead to either a Ras-dependent or -independent phosphorylation of ERK1/2 MAPK. The Ras-dependent pathway may also be activated by the βγ G-protein subunits that have been previously reported to activate the Ras/ERK1/2 MAPK pathway. In addition, this study has shown that PI3K is partially involved in CCL1-mediated

ERK1/2 phosphorylation, which suggests that the PI3K-mediated Ras-dependent pathway can be utilised but appears not to be essential. This study has also demonstrated that PI3K is required, in addition to PKC, for CCL1-mediated PKB phosphorylation. In accordance with previously published studies, CCL1 could induce phosphorylation of PKB in a PLC- and PKC-dependent pathway (Figure 30). Finally, the data presented in this study reveals that CCL1-mediated chemotaxis of HUT-78 cells requires PI3K, PKC, PLC and calcium for an optimal chemotactic response (Figure 31).

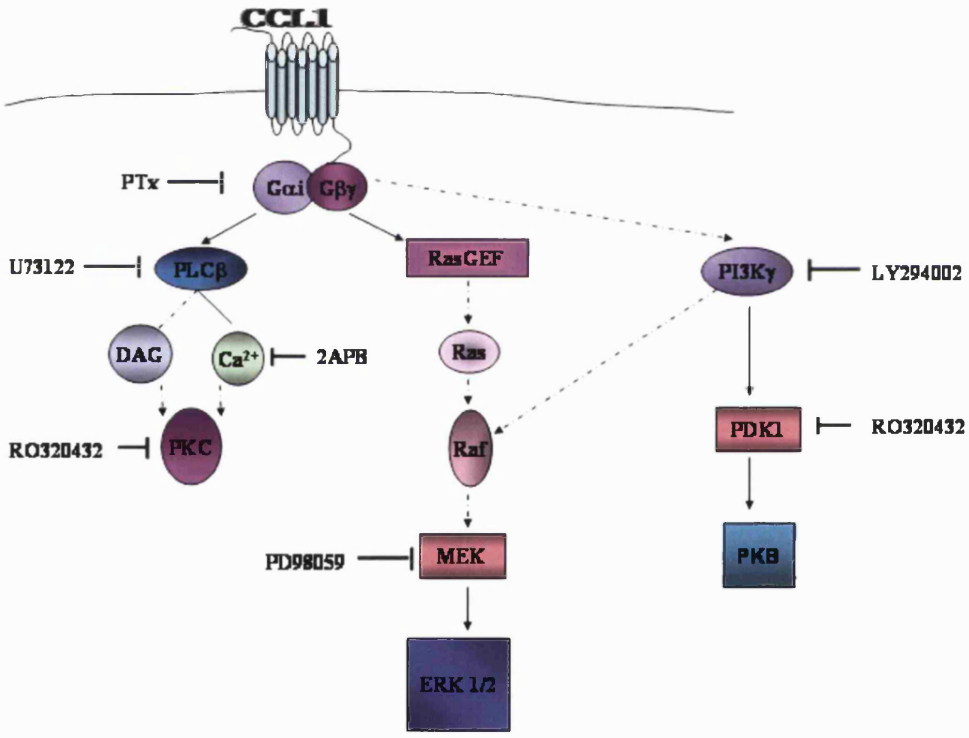

Figure 30. A Model of the Signalling Pathways mediated by CCR8/CCL1

The solid lines represent pathways which have been shown in this study to be involved in CCL1-mediated responses. The dotted lines show potential pathways that have been demonstrated in different systems as outlined in chapter 1.

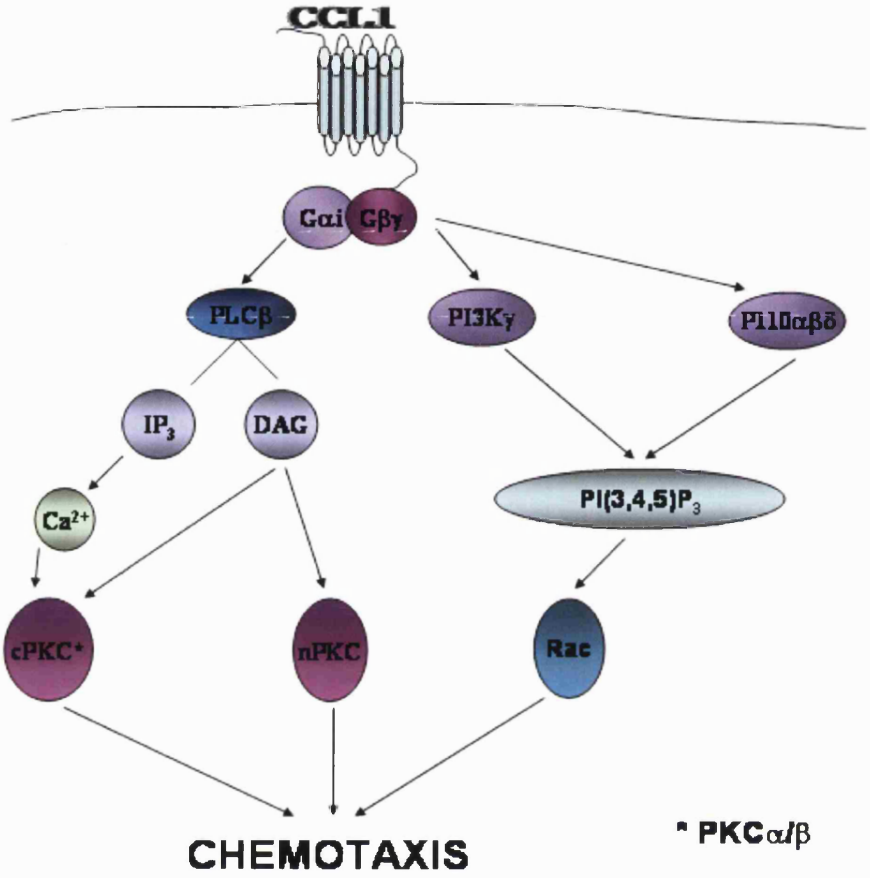

Figure 31. A Model of the Signalling Pathways in CCL1-mediated Chemotaxis

Proposed routes are shown by which PI3Kγ and p85/p110αβδ PI3K may contribute to CCL1-stimulated functional events via 3'-phosphoinositide lipid dependent and/or protein serine kinase-dependent activity.

Chapter 5

Chapter 5

Results: The Role of SHIP in Regulating Chemokine-mediated Responses

5.1 Introduction

B cells express receptors that mediate positive and inhibitory signals within the cell, namely the BCR and FcγRIIb receptor respectively (Figure 32). Stimulation of the BCR with antigen results in proliferation and secretion of soluble antigen-specific immunoglobulin. Co-ligation of the BCR and FcγRIIb receptor by immune complexes down-regulates BCR-mediated responses and prevents excess immunoglobulin production (382,383). The function of the FcγRIIb receptor therefore prevents over-reaction of immune responses and protects against autoimmunity (384,385). B cells also express a small subset of chemokine receptors that guide the movements of re-circulating B cells within lymphoid tissues and include CXCR5, CCR7 and CXCR4 (386).

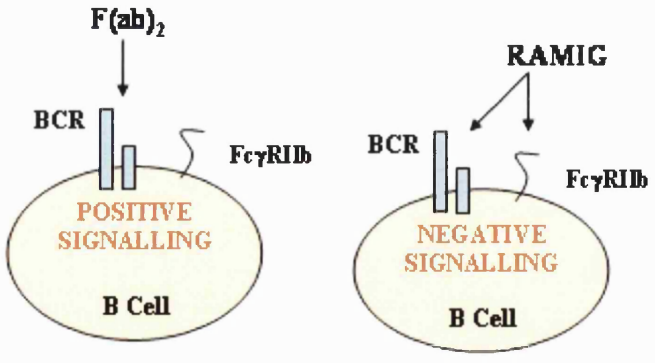

Figure 32 Positive and Negative Signalling in B Cells

Previous research has shown that the FcγRIIb-mediated inhibitory effects are largely dependent on the 5' lipid phosphatase SHIP, where SHIP catalyzes removal of the 5-phosphate of $PI(3,4,5)P_3$ and thus reduces the levels of $PI(3,4,5)P_3$ within the cell. The purpose of this study was to investigate whether the FcγRIIb-mediated recruitment/activation of SHIP is able to regulate $PI(3,4,5)P_3$ accumulation and $PI(3,4,5)P_3$-dependent biochemical and functional events elicited by G protein-coupled chemokine receptors. The GPCR investigated in this section of the study was the chemokine receptor CXCR4, since it is constitutively expressed on lymphoid cells. In addition, it is the best characterised chemokine receptor, so tools and reagents are readily available for its biochemical and functional analysis.

The functional role of SHIP in the regulation of BCR-mediated responses was first examined using a B cell model in which the effects of co-ligating the BCR and FcγRIIb receptor on BCR-mediated responses were examined. Co-ligation of the BCR and FcγRIIb receptor was shown to attenuate BCR-mediated calcium mobilisation (387), ERK1/2 (225) and PKB phosphorylation (216) The initial experiments were designed to verify that co-ligation of the BCR and FcγRIIb receptor resulted in (i) the tyrosine phosphorylation of SHIP, and (ii) impaired BCR-mediated responses, such as calcium mobilisation, $PI(3,4,5)P_3$ accumulation and ERK1/2 and PKB phosphorylation in the B lymphoma cell line A20. The aim was then to determine if the FcγRIIb-mediated tyrosine phosphorylation of SHIP inhibited the responses induced by independently activated receptors, such as the chemokine

receptor CXCR4. This study also utilized a Jurkat T cell model that expresses a tetracycline-regulated, constitutively active SHIP mutant, in order to study the direct effects of SHIP activity on CXCL12-mediated responses.

In this study, $F(ab')_2$ fragments of rabbit anti-mouse IgG (RAMIG) were used to stimulate the murine BCR and intact RAMIG was used to co-ligate the BCR with the FcγRIIb receptor.

Co-aggregation of the BCR and FcγRIIb Receptor Blocks Downstream Responses in A20 cells

Engagement of the BCR with antigen induces the PI3K-dependent accumulation of $PI(3,4,5)P_3$ leading to the recruitment of PH domain-containing proteins such as the protein tyrosine kinase, Btk. Phosphorylation and activation of PLCγ by Btk results in the hydrolysis of $PI(4,5)P_2$ generating IP_3 and DAG. The release of calcium, in response to IP_3/IP_3R ligation, regulates many cellular processes, including gene transcription, proliferation and antibody production. To verify that co-ligation of the BCR and FcγRIIb receptor inhibits BCR-mediated calcium mobilisation, A20 cells were pre-loaded with Fura-2 (5 μM) for 30 minutes and then stimulated with either 10 μg/ml of $F(ab')_2$ fragments of RAMIG or 15 μg/ml of intact RAMIG. These results show that stimulation of the BCR alone with $F(ab')_2$ fragments of RAMIG leads to an increase of intracellular Ca^{2+} from a basal level of 100 nM to 1000 nM.

The BCR-induced Ca^{2+} signal was notably inhibited following co-ligation of the BCR and FcγRIIb receptor with intact RAMIG (Figure 33).

Recruitment of SHIP to FcγRIIb and its subsequent activation following co-ligation of the BCR and FcγRIIb receptor has been reported (221,383). A20 cells were stimulated at intervals between 1 and 60 minutes with either 10 μg/ml of $F(ab')_2$ fragments of RAMIG or 15 μg/ml of intact RAMIG. Western blot analysis of anti-SHIP immunoprecipitates, probed with an anti-phospho tyrosine antibody, 4G10, revealed a modest and transient tyrosine phosphorylation of SHIP at 5 minutes following activation of the BCR alone. However, subsequent to co-ligation of the BCR and FcγRIIb receptor with intact RAMIG, the tyrosine phosphorylation of SHIP was shown to be prolonged and more robust between 1 and 30 minutes (Figure 34).

Previous reports have shown that tyrosine phosphorylation of SHIP leads to an enhanced catalytic activity of SHIP, that is the ability of SHIP to reduce $PI(3,4,5)P_3$ to $PI(3,4)P_2$ (388). To verify that the enhanced tyrosine phosphorylation of SHIP correlated with increased catalytic activity following BCR and FcγRIIb receptor co-ligation, $PI(3,4,5)P_3$ levels and PKB phosphorylation were monitored. Since PKB is fundamentally dependent on $PI(3,4,5)P_3$ accumulation, then inhibition of $PI(3,4,5)P_3$ accummulation should be reflected in the attenuation of PKB phosphorylation. A20 cells stimulated for 1 minute with $F(ab')_2$ fragments of RAMIG led to an increase of $PI(3,4,5)P_3$ above control. This response was abrogated following the co-ligation of

the BCR and FcγRIIb receptor with intact RAMIG (Figure 35). Similarly, stimulation of A20 cells with $F(ab')_2$ fragments of RAMIG for 5 minutes resulted in a marked phosphorylation of PKB above control levels, yet stimulation of cells with intact RAMIG reduced this response to basal levels (Figure 36).

In addition to its catalytic activity, SHIP has been reported to function as an adapter. The SH2 domain of SHIP is thought to interact with the PTB domain of Shc, displacing the Shc/Grb2 complex and thus uncoupling the Ras pathway from BCR signalling (389). This section of the study focusses on the Ras/MAPK pathway to verify if co-ligation of the BCR and FcγRIIb receptor would have similar inhibitory effects as seen on the PI3K pathway. Once again, A20 cells were stimulated for 5 minutes with either $F(ab')_2$ fragments of RAMIG or intact RAMIG. Since phosphorylation of ERK1/2 is necessary for its activation, a phospho-ERK1/2 antibody was used to monitor ERK1/2 phosphorylation as an indirect marker of ERK activation. Using this approach, it was apparent that 5 minute stimulation with $F(ab')_2$ fragments of RAMIG resulted in a robust phosphorylation of ERK1/2, compared to a diminished response following co-ligation of the BCR and FcγRIIb receptor (Figure 36).

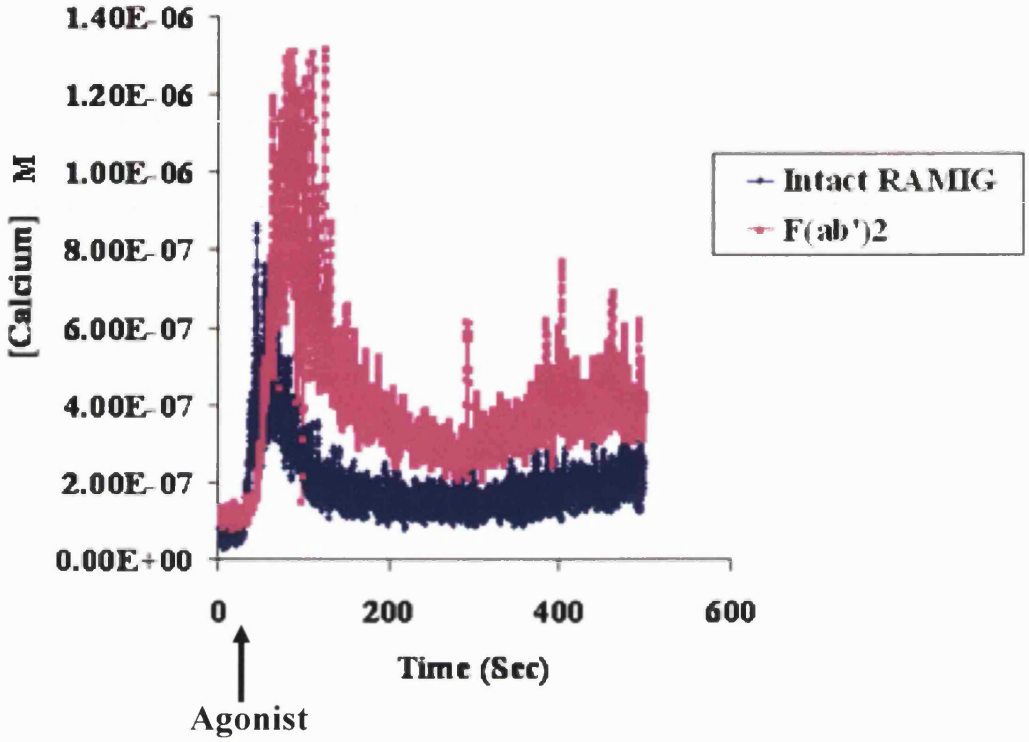

Figure 33. Co-ligation of the BCR and FcγRIIb Receptor Inhibits the Intracellular Ca^{2+} Mobilisation in A20 cells

Intracellular free calcium concentration ($[Ca^{2+}]_i$) of A20 cells was determined by pre-loading the cells ($2x10^6$/ml/sample) with 5 µM of Fura-2 AM at 37°C for 30 minutes. Cells were washed free of excess Fura-2 using HBSS buffer supplemented with 100 µM Ca^{2+} and 1 mM Mg^{2+} and re-suspended at $2x10^6$/ml. Fura-loaded cells were aliquoted into a 2-ml cuvette. To the cell suspension 20 µl of a 100 mM calcium chloride stock (final [1 mM]) was added and allowed to equilibrate at 37°C in the spectrofluorimeter for 5 minutes. Prior to the addition of the agonists, a basal Ca^{2+} measurement was taken for 30 seconds. The agonist-induced responses, with either intact RAMIG (15 µg/ml) or $F(ab')_2$ fragments of RAMIG (10 µg/ml) were monitored for at least 500 seconds and were detected on the spectrofluorimeter (Photon Technologies). Data are from a single experiment representative of 2 others.

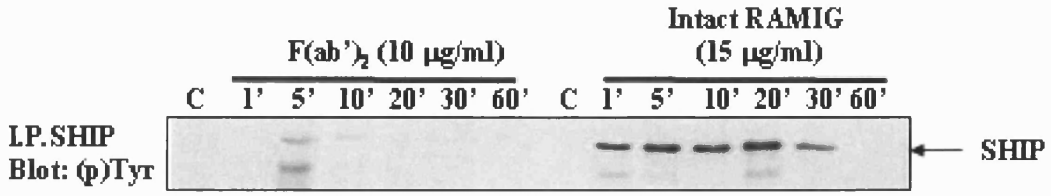

Figure 34. SHIP is a Target of BCR-activated Phosphotyrosine Kinases.

A20 cells were aliquoted in 0.5 ml at $1x10^7$cells/ml and either left unstimulated (C) or stimulated for the indicated time with either 10 µg/ml of $F(ab')_2$ fragments of RAMIG or 15 µg/ml of intact RAMIG. SHIP was subsequently immunoprecipitated from cell lysates using a goat anti-SHIP polyclonal antibody. Immunoblotting with the anti-phosphotyrosine antibody 4G10 was used to detect tyrosine phosphorylated SHIP. Data are from a single experiment representative of 2 others.

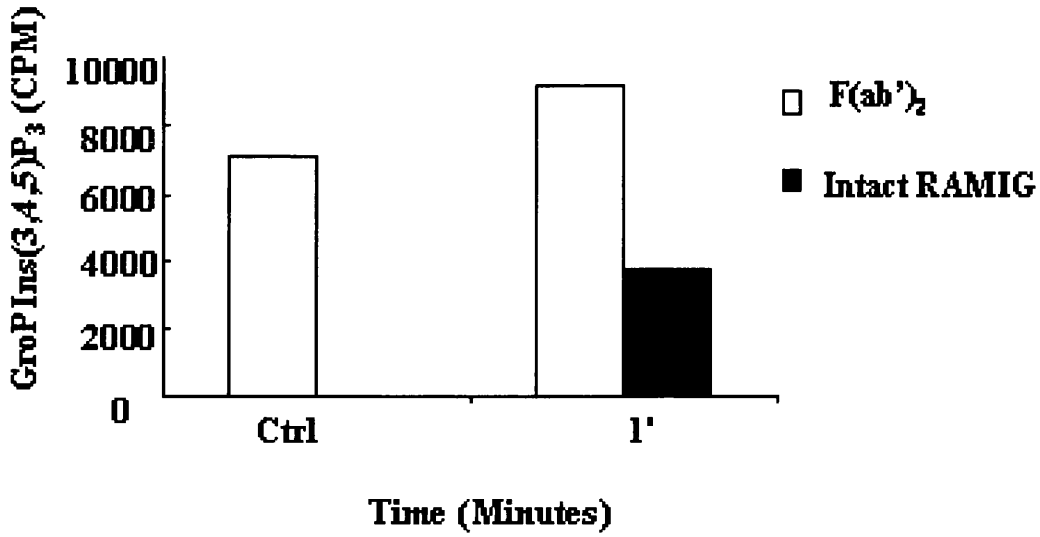

Figure 35. Co-ligation of BCR and FcγRIIb Receptor Reduces the Levels of PI(3,4,5)P_3 in A20 Cells.

A20 ($2x10^7$/ml) cells were labelled with [^{32}P] orthophosphoric acid for 1.5 h. Following the removal of un-incorporated label, cells ($2x10^6$/ml) were either left unstimulated (Ctrl) or stimulated with either F(ab')$_2$ fragments of RAMIG or intact RAMIG for 1 minute. Reactions were terminated with chloroform/methanol/water, and phospholipids extracted, deacylated and fractionated by HPLC. Data are from a single experiment representative of 3 others.

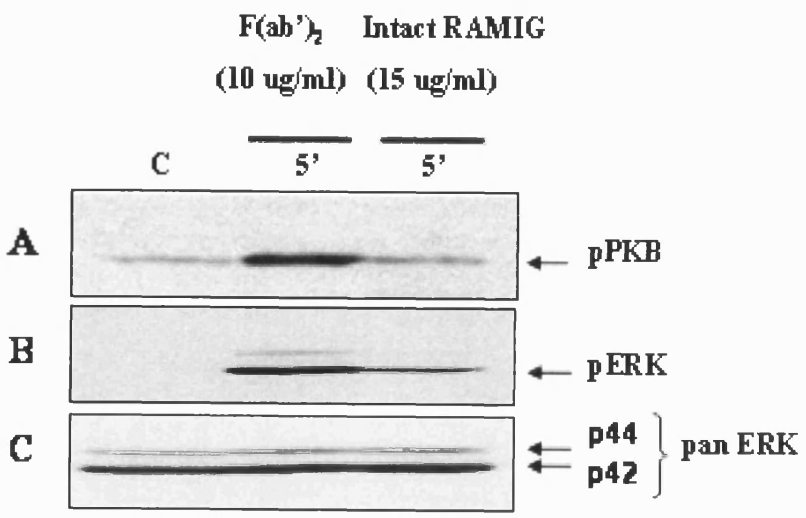

Figure 36. Co-ligation of the BCR and FcγRIIb Receptor Reduces BCR-mediated PKB and ERK1/2 phosphorylation in A20 Cells.

A20 cells were aliquoted in 0.5 ml at $2x10^6$cells/ml and left unstimulated (C) or stimulated for 5 minutes with either 10 µg/ml of $F(ab')_2$ fragments of RAMIG or 15 µg/ml of intact RAMIG. The equivalent to $2x10^5$ cells of each sample was separated by SDS-PAGE. Immunoblotting with either the Ser^{473} PKB (A) or p44/42 phosphospecific ERK1/2 (B) antibody was used to detect phosphorylated PKB and ERK1/2. The immunoblot was stripped and reprobed with the pan-ERK antibody that recognises both phosphorylated and non-phosphorylated forms of ERK to allow parity of loading to be examined (C). Data are from a single experiment representative of 2 others.

SHIP is Not a Target of CXCL12-activated Phospho-tyrosine Kinases

The next step was to establish whether CXCL12-mediated responses led to the tyrosine phosphorylation of SHIP and whether stimulation of CXCR4 influenced the kinetics of FcγRIIb-mediated tyrosine phosphorylation of SHIP. A20 cells were either stimulated with CXCL12 alone for the indicated times, or with intact RAMIG in addition to CXCL12. Western blot analysis of SHIP immunoprecipitates using anti-phosphotyrosine antibodies revealed that SHIP was not tyrosine phosphorylated following stimulation of CXCR4 alone and that FcγRIIb-mediated tyrosine phosphorylation of SHIP was not affected following stimulation of CXCR4 (Figure 37).

Co-ligation of the BCR and FcγRIIb Receptor Abrogates CXCL12-stimulated ERK1/2 and PKB phosphorylation in A20 Cells

To investigate whether FcγRIIb-activated SHIP can inhibit biochemical signals elicited by CXCR4, the effects of co-ligating the BCR and FcγRIIb receptor on CXCL12-mediated PKB and ERK1/2 phosphorylation were examined. The data presented in Figure 37 show that stimulation of A20 cells with 10 nM of CXCL12 for 2 minutes induced a robust PKB and ERK1/2 phosphorylation (lane 2), compared to the unstimulated control (lane 1). Co-ligation of the BCR and FcγRIIb receptor resulted in the phosphorylation of PKB and ERK1/2 at low concentrations but higher

concentrations resulted in a gradually diminished signal (lanes 3-7). This is presumably because low concentrations of RAMIG would bind predominantly to the BCR, whereas higher concentrations also bind to the lower affinity FcγRIIb receptor, which elicits inhibitory effects that counteract the positive signals from the BCR. In contrast, activation of the BCR alone with $F(ab')_2$ fragments of RAMIG showed a concentration-dependent PKB and ERK1/2 phosphorylation (lanes 13-17). Pre-incubation of cells for 30 minutes with 150 μg/ml of intact RAMIG, which failed to elicit significant phosphorylation of PKB/ERK1/2 above basal levels, followed by stimulation with 10 nM of CXCL12 for 2 minutes, resulted in the inhibition of both PKB and ERK1/2 phosphorylation (lane 12), when compared to stimulation with CXCL12 alone (lane 2) (Figure 38).

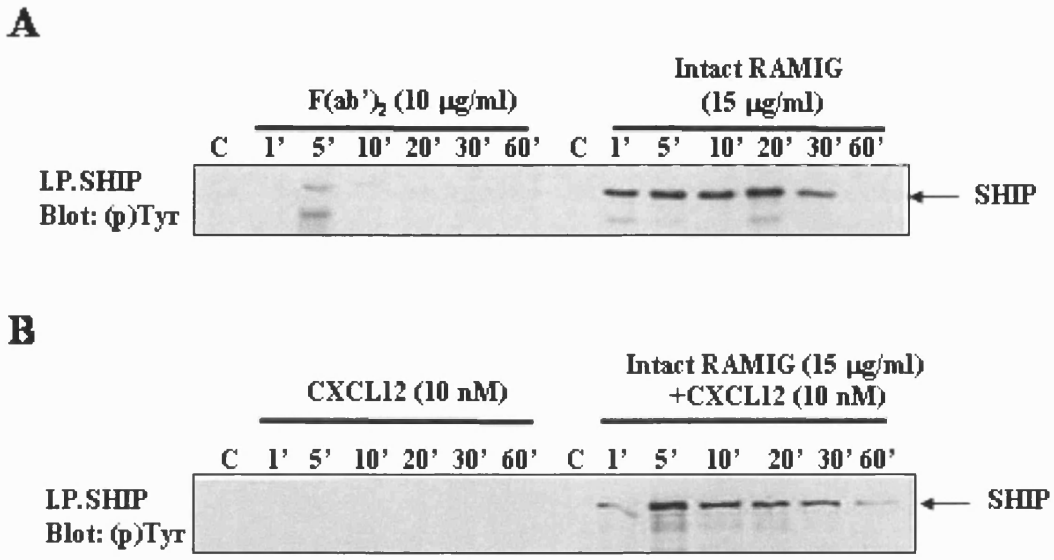

Figure 37. SHIP is Not a Target of CXCL12-activated Phospho-tyrosine Kinases

A20 cells were aliquoted in 0.5 ml at $1x10^7$cells/ml and either left unstimulated (C) or stimulated for the indicated time with either 10 µg/ml $F(ab')_2$ or 15 µg/ml intact RAMIG (A), or either 10 nM CXCL12 or 15 µg/ml of intact RAMIG and 10 nM CXCL12 (B). (Figure 35A is the same experiment as Figure 32 and is duplicated here for ease of analysis.) SHIP was subsequently immunoprecipitated from cell lysates using a goat anti-SHIP polyclonal antibody. Immunoblotting with the anti-phosphotyrosine antibody 4G10 was used to detect tyrosine phosphorylated SHIP. Data are from a single experiment representative of 2 others.

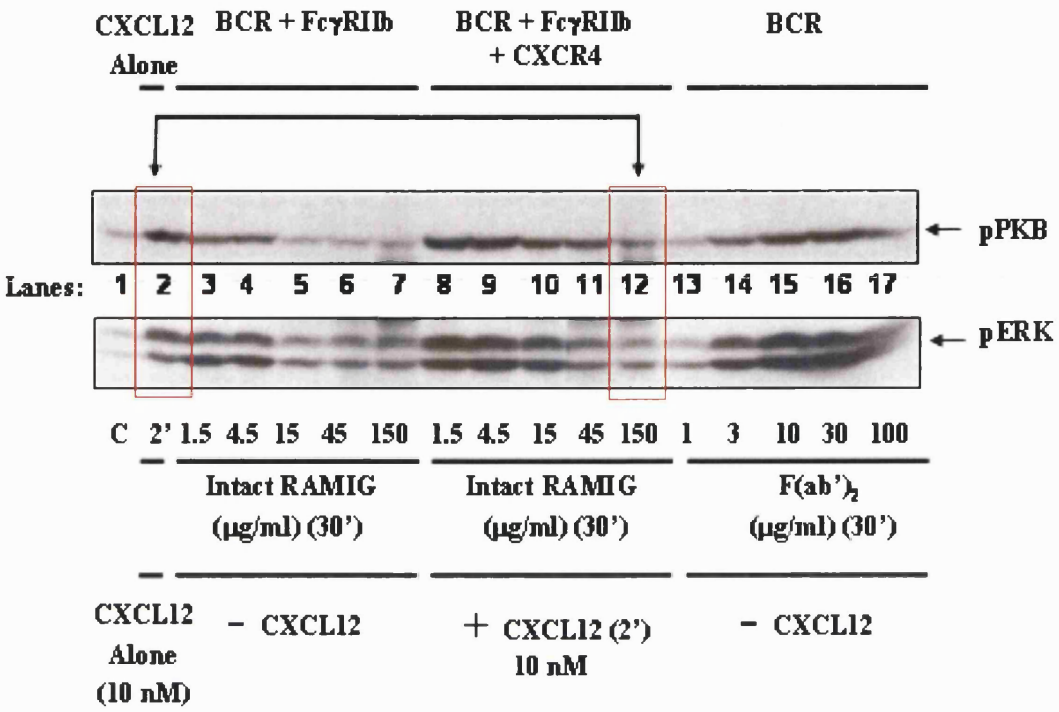

Figure 38. Co-aggregation of the BCR and FcγRIIb Receptor Reduces CXCL12-stimulated Phosphorylation of PKB and ERK1/2.

A20 cells ($2x10^6$/ml) were either left unstimulated, (C) *(lane 1);* or stimulated with either 10 nM of CXCL12 for 2 minutes *(lane 2),* or 1.5-150 μg/ml of intact RAMIG for 30 minutes *(lanes 3-7),* or 1-100 μg/ml of $F(ab')_2$ fragments of RAMIG for 30 minutes *(lanes 13-17).* Additionally, some cells were pre-incubated with RAMIG for 30 minutes prior to addition of 10 nM CXCL12 for a further 2 minutes. The equivalent to $2x10^5$ cells of each sample was separated by SDS-PAGE. Immunoblotting with either the p44/42 phosphospecific ERK1/2 or Ser^{473} PKB antibody were used to detect phosphorylated ERK1/2 and PKB. Data are from a single experiment representative of 2 others.

The Effect of Expressing a Constitutively Active SHIP Mutant on CXCL12-mediated PI(3,4,5)P_3 levels and PKB Phosphorylation in the Jurkat Leukaemic T cell Line

Having demonstrated that tyrosine phosphorylation of SHIP in B cells correlates with a reduction of CXCL12-mediated PKB and ERK1/2 phosphorylation (Figure 38), attention was turned to a T cell model which expresses a tetracycline-regulated, constitutively active SHIP mutant. The Jurkat T cell line, which does not normally express either phosphatases SHIP or PTEN (388), were stably transfected with a chimeric protein which contained the phosphatase core of SHIP-1 fused to the extracellular/transmembrane region of the rat CD2 (CD2:SHIP) (293). The resulting membrane-localised SHIP catalytic domain was characterised as exhibiting constitutive 5'-inositol lipid phosphatase activity. As a negative control Jurkat cells were also transfected with a point-mutated version of SHIP-1, in which the cysteine residue at position 701 within the catalytic signature motif was mutated to alanine (CD2:C^{701}ASHIP). This mutant retains some activity, although it is at least 10-fold less active than CD2:SHIP (293).

CXCL12 stimulation experiments were conducted in CD2:SHIP clones in the presence or absence of expression (i.e., precultured without or with tetracycline, respectively). In response to CXCL12 stimulation, increases in PI(3,4,5)P_3 above basal were detected after 30 seconds in nonexpressing, CD2:SHIP clones (i.e., cells

incubated in the presence of tetracycline). The expression of CD2:SHIP (i.e., clones incubated in the absence of tetracycline) led to a 33% inhibition of CXCL12-stimulated $PI(3,4,5)P_3$ (Figure 39). The inhibition of chemokine-stimulated $PI(3,4,5)P_3$ by CD2:SHIP confirms that this construct does indeed disrupt $PI(3,4,5)P_3$, by reducing $PI(3,4,5)P_3$ to $PI(3,4)P_2$. The extent of PI3K activation can also be assessed indirectly by monitoring the phosphorylation of the serine/threonine kinase PKB, whose recruitment and subsequent activation is entirely dependent on D-3 phosphoinositide lipid accummulation (167). CXCL12 stimulation time-course experiments were conducted in CD2:SHIP and CD2:C^{701}ASHIP clones in the presence or absence of expression. CXCL12-mediated increases in PKB phosphorylation above basal were detected after 1 minutes in nonexpressing, CD2:SHIP clone (Figure 40(i), lanes 1, 3, 5, 7 and 9). The expression of CD2:SHIP led to an inhibition of basal and CXCL12-stimulated PKB phosphorylation (Figure 40(i), lanes 2, 4, 6, 8, and 10). CXCL12-mediated increases in PKB phosphorylation above basal were detected after 2 minutes in nonexpressing, CD2:C^{701}ASHIP clones (Figure 40(ii), lanes 1, 3, 5, 7 and 9). However, expression of CD2:C^{701}ASHIP did not result in the inhibition of CXCL12-mediated PKB phosphorylation (Figure 40(ii), lanes 2, 4, 6, 8, and 10). CD2:SHIP and CD2:C^{701}ASHIP expression was assessed by probing immunoblots with a rCD2 antibody (Figure 40(ii)).

The Effect of a Constitutively Active SHIP mutant on CXCL12-mediated Chemotaxis

PI3Ks and their lipid product PI(3,4,5)P_3 have been widely implicated in controlling cell migration and polarity (390). During leukocyte chemotaxis, type IA PI3Ks are required for lamellipodium extension and migration towards colony-stimulating factor 1 (CSF-1) (391), whereas type IB PI3Ks are required for neutrophil chemotaxis to a variety of inflammatory mediators that signal via GPCRs (201,392,393). Moreover, optimal chemotactic response of leukemic T cells to CXCL12 requires the activation of both class IA and class IB PI3K (287). On the understanding that SHIP can de-phosphorylate PI(3,4,5)P_3 to PI(3,4)P_2, it would be plausible to speculate that SHIP may inhibit the directional movement of cells in response to a given chemoattractant stimulus. Experiments were designed to determine whether SHIP plays a role in the regulation of CXCL12-mediated chemotaxis. These data showed that chemotaxis of SHIP-expressing Jurkat cells (CD2:SHIP) in response to CXCL12 was markedly inhibited compared to cells not expressing SHIP. However, cells expressing the inactive form of SHIP (CD2:C701A SHIP) responded to CXCL12 with a similar chemotactic index compared to those not expressing the inactive mutant (Figure 41).

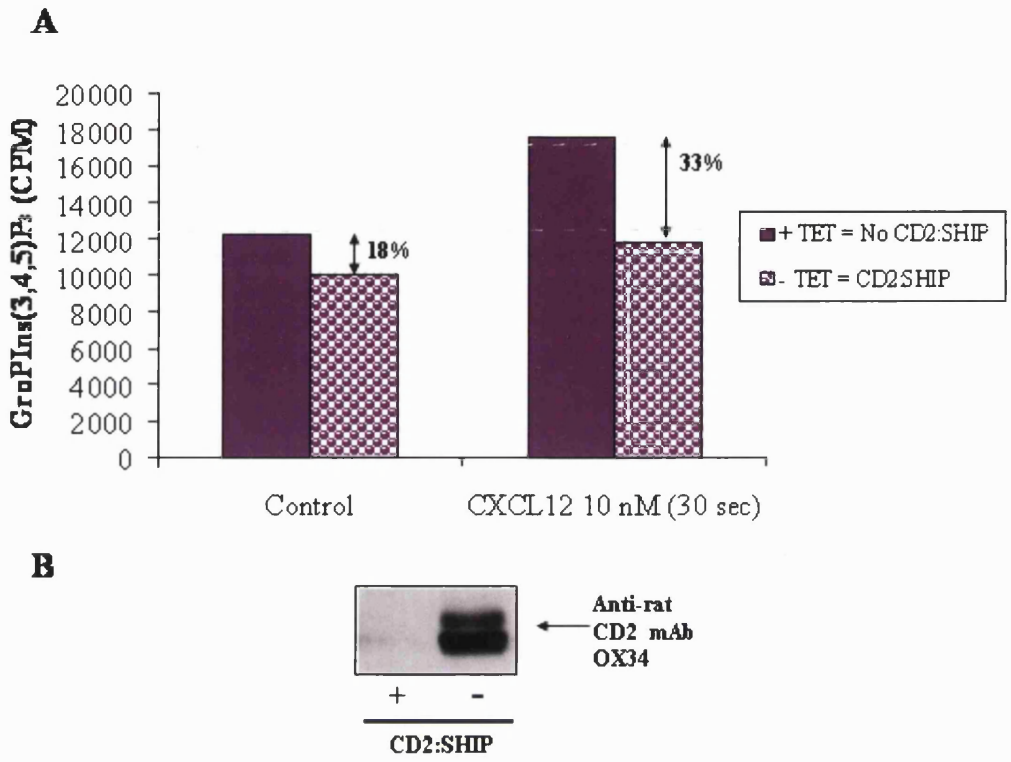

Figure 39. The Effect of a Constitutively Active SHIP mutant on CXCL12-mediated Lipid Accumulation

Jurkat cells stably transfected with CD2:SHIP were cultured overnight with or without Tetracycline (TET) (2 μg/ml) and subsequently labelled with [^{32}P] orthophosphoric acid for 4 h. Following the removal of un-incorporated label, cells ($2x10^7$/ml) were stimulated with CXCL12 (10 nM) for 30 seconds. Reactions were terminated with chloroform/methanol/water, and phospholipids extracted, deacylated and fractionated by HPLC. 18 and 33% represents the percentage inhibition of $PI(3,4,5)P_3$ accumulation caused by expressing CD2:SHIP. The percentage inhibition of CD2:SHIP expression are shown on the graph (A). Samples of cells prior to labelling were lysed and western blotted with anti-rCD2 mAb OX34 to confirm the expression of the construct upon removal of tetracycline (B). These data are from a single experiment representative of 3 others.

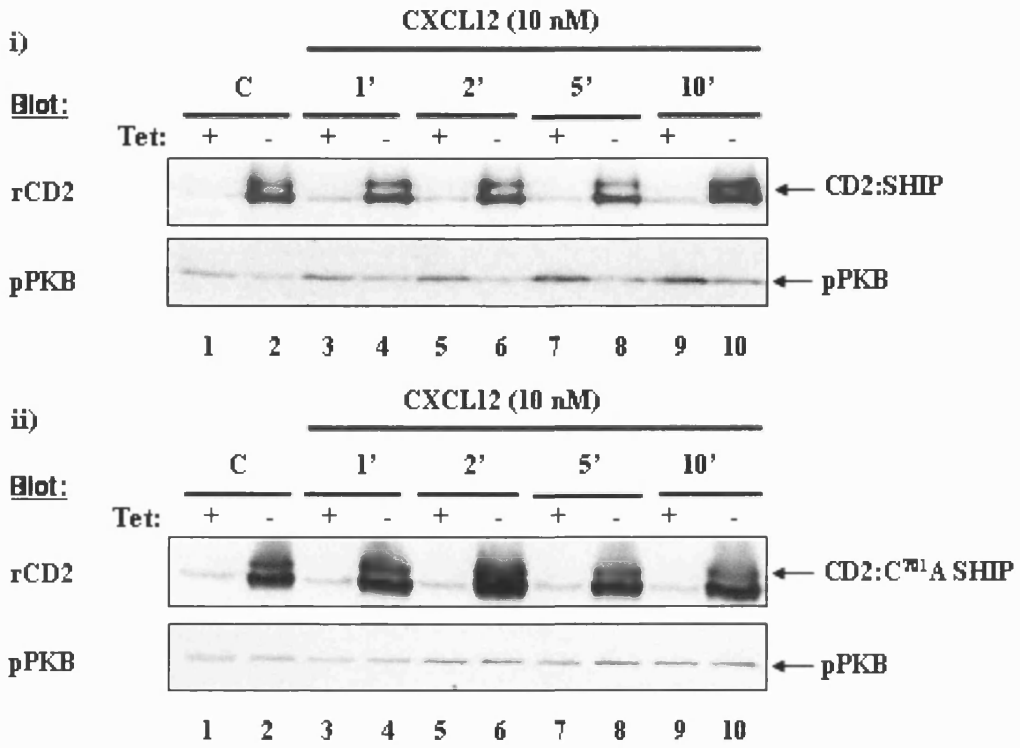

Figure 40. The Effect of a Constitutively Active SHIP Mutant on CXCL12-mediated PKB phosphorylation.

Jurkat cells, stably transfected with CD2:SHIP (i) or CD2:C^{701}A SHIP (ii), were cultured overnight either with *(+, lanes 1, 3, 5, 7 and 9)* or without *(-, lanes 2, 4, 6, 8 and 10)* tetracycline (tet) (2 μg/ml). 0.5 ml aliquots at $2x10^6$cells/ml were either left unstimulated (C) or stimulated with 10 nM CXCL12 for the times indicated. The equivalent to $2x10^5$ cells of each sample was separated by SDS-PAGE. Immunoblotting with either the anti-rCD2 (*top panel*) or Ser^{473} PKB (*bottom panel*) antibody was used to determine the expression of CD2:SHIP/CD2:C^{701}ASHIP or to detect phosphorylated PKB. These data are from a single experiment representative of 2 others.

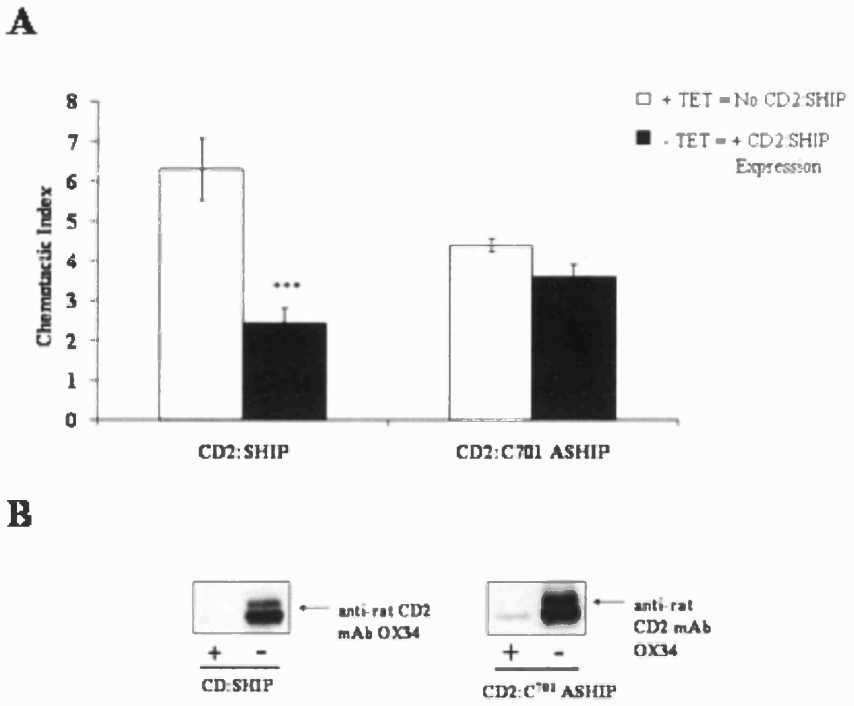

Figure 41. The effect of a Constitutively Active SHIP mutant on CXCL12-mediated Chemotaxis

Jurkat cells transfected with either the CD2:SHIP or CD2:C^{701} ASHIP construct were incubated for 24 h in media in the presence and absence of tetracycline (Tet). Cells ($1x10^6$/ml) were subsequently aliquoted in a 96-well chemotaxis chamber along with 10 nM CXCL12 and incubated at 37°C for 1.5 h. Results are expressed as a mean chemotactic index (+/- SEM), which is the ratio of OD readings of the stimulated samples against the OD readings of the control samples incubated with medium alone, from quadruplicate wells (A). Data were analysed by Student's *t* test to compare responses in the presence and absence of SHIP expression (***, $p < 0.00005$). Samples of cells from the chemotaxis experiment were lysed and western blotted with anti-rCD2 mAb OX34 to confirm the expression of the construct upon removal of tetracycline (B). These data are from a single experiment representative of 3 others.

Summary of Findings

- Co-ligation of the BCR and FcγRIIb receptor resulted in a prolonged and more robust tyrosine phosphorylation of SHIP compared to that seen following activation of the BCR alone in A20 cells.

- The augmented tyrosine phosphorylation of SHIP directly correlated with a reduction in lipid accumulation as well as in the phosphorylation of both ERK1/2 and PKB in A20 cells.

- CXCL12 stimulation did not induce tyrosine phosphorylation of SHIP in A20 cells.

- Concurrent activation of FcγRIIb receptor and CXCR4 did not influence the kinetics of the tyrosine phosphorylation of SHIP in A20 cells.

- Co-ligation of the BCR and FcγRIIb receptor led to the inhibition of CXCL12-stimulated ERK1/2 and PKB phosphorylation in A20 cells.

- Using a leukaemic T cell line which expresses an inducible constitutively active SHIP mutant (CD2:SHIP), we demonstrated that the induction of SHIP expression led to a substantial inhibition of CXCL12-stimulated PKB phosphorylation, lipid accumulation and chemotaxis. These effects were not observed in T cells expressing an inactive form of SHIP (CD2:C^{701} ASHIP).

5.2 Discussion

The FcγRIIb Receptor Regulates BCR-mediated Responses

Many studies have demonstrated that co-ligation of the BCR and FcγRIIb receptor results in the recruitment of SHIP and subsequent reduction in PI3K-dependent, BCR-mediated responses, such as antibody production and proliferation (221,383,394,395). The catalytic function of SHIP converts PI3K-dependent $PI(3,4,5)P_3$ to $PI(3,4)P_2$ and thus prevents the binding of PH domain-containing proteins like Btk and PKB from binding to $PI(3,4,5)P_3$.

In view of the fact that chemokines are known to induce PI3K-dependent responses, we aimed to determine whether a reduction in FcγRIIb-mediated $PI(3,4,5)P_3$ by SHIP would inhibit biochemical and functional responses elicited by chemokines. Using the B lymphoma cell line A20 as a model, the effects of co-ligating the BCR and FcγRIIb receptor were first examined to validate our system. The data presented in this section verified that co-ligation of the BCR and FcγRIIb receptor attenuated BCR-mediated calcium mobilisation, $PI(3,4,5)P_3$ accumulation and PKB and ERK1/2 phosphorylation. Similarly, co-ligation of the BCR and FcγRIIb receptor was also found to inhibit CXCL12-mediated PKB and ERK1/2 phosphorylation.

The second model used in this study to establish whether SHIP can regulate chemokine signalling and chemotaxis was the Jurkat T cell model, which expresses a tetracycline-regulated, constitutively active SHIP mutant. SHIP-expressing Jurkat cells were found to inhibit CXCL12-mediated $PI(3,4,5)P_3$ accumulation, PKB phosphorylation and cell migration, compared to non-expressing cells.

Co-ligation of the BCR and FcγRIIb Receptor Inhibits CXCL12-mediated ERK1/2 and PKB Phosphorylation

The interaction of SHIP with the ITIM motif of the FcγRIIb receptor is well documented by D'Ambrosio *et al* (1996) (212) and Fong *et al* (1996) (213), although the effects SHIP may exert on other independently activated receptors is ill defined. It is evident from the results of this research that activation of SHIP is dependent on the co-ligation of the BCR and FcγRIIb receptor, as assessed by the inability of CXCL12 to induce the tyrosine phosphorylation of SHIP. Moreover, co-ligation of the BCR and FcγRIIb receptor prior to CXCL12 stimulation did not influence the tyrosine phosphorylation of SHIP following co-ligation alone. In contrast, ligation of the FcγRIIb receptor prior to CXCL12 stimulations, led to the inhibition of both ERK1/2 and PKB when compared to CXCL12 responses alone.

We have been unable to demonstrate a direct association between CXCR4 and SHIP, however, SHIP may yet interact with CXCR4 via its SH2 domain and the failure to

detect CXCR4-SHIP complexes may be explained if this association occurs at very low stoichiometry and thus, below the detectable limits of the immunoblotting assay used in this study. Alternatively, the CXCR4-SHIP associations may be of low affinity, such that they are easily disrupted by the NP40 detergent present in the cell lysis buffer used in this study.

Given the lack of a recognised SHIP-binding motif within the CXCR4 cytoplasmic tail, it is possible that CXCR4 couples to SHIP via intermediate adaptor molecules. The presence of proline-rich domains, SH2 domains and binding sites for phosphotyrosine binding domains within SHIP gives it the potential for diverse biochemical interactions with a plethora of other proteins. If such protein complexes do exist, they may occur very transiently and at low stoichiometry and/or affinity such that they may not be maintained or detected in the CXCR4 immune complexes using the approaches described in this study. Although the mechanisms by which CXCR4 interacts with SHIP remain unclear, CXCR4 is reported to activate the protein tyrosine phosphatase (PTP) SHP2 (396). Tyrosine-phosphorylated SHP2 can act as an adaptor molecule and has been shown to bind to SHIP as well as several of the src-related tyrosine kinases, lyn and fyn (397-399). Association of SHIP with SHP2 occurs through the direct interaction of the SH2 domain of SHIP with a pYXN(I/V) sequence within SHP2 (398). Hence, it is possible that SHP2 may be involved in mediating tyrosine phosphorylation of either intermediate proteins or SHIP in response to CXCR4 ligation.

Although the CXCR4-stimulated increase in enzyme activity present in SHIP immunoprecipitates correlates with tyrosine phosphorylation, a requirement for tyrosine phosphorylation in SHIP activation remains to be established. Nevertheless the data from this study appear to contradict observations in other systems where SHIP has been reported to be negatively regulated by tyrosine phosphorylation (400). Moreover, the enzymatic activity of SHIP immunoprecipitated from growth factor-stimulated cells is the same as that derived from unstimulated cells, but if SHIP is immunoprecipitated from these cells with anti-phosphotyrosine or anti-Shc antibodies, then much more enzyme activity can be obtained from growth factor-stimulated cells (401). Our data indicate that T cells may possess an entirely different means for SHIP activation after CXCR4 ligation, although at present what function the tyrosine phosphorylation of SHIP serves is still unclear. One possibility is that tyrosine phosphorylation of SHIP is required for its relocalisation to the plasma membrane, since phosphotyrosine residues may mediate interaction with adaptor proteins.

The Mechanisms which Lead to FcγRIIb-dependent Activation of SHIP

It is possible that the inhibition of CXCL12-mediated, PI3K-dependent PKB phosphorylation occurs through a similar mechanism to that described for BCR-mediated responses by Carver *et al* (2000) (218), where PKB is prevented from

relocating to the membrane as a result of SHIP-mediated $PI(3,4,5)P_3$ degradation (Figure 42).

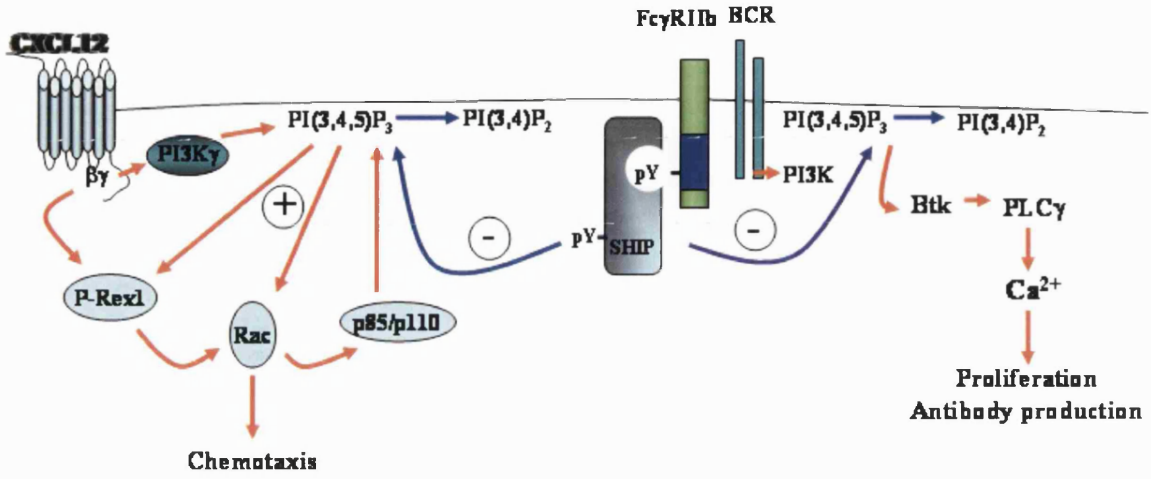

Figure 42. The Catalytic Activity of SHIP Reduces the Levels of $PI(3,4,5)P_3$ in the Cell

Co-ligation of the BCR and FcγRIIb receptor leads to the recruitment of SHIP to the ITIM motif of FcγRIIb and subsequent reduction in $PI(3,4,5)P_3$. This catalytic role of SHIP inhibits BCR-mediated, PI3K-dependent responses such as proliferation and antibody production. FcγRIIb-mediated reduction of $PI(3,4,5)P_3$ by SHIP, may also inhibit PI3K-dependent responses mediated by independently activated receptors such as chemokine receptors. For instance, $PI(3,4,5)P_3$ plays a central role in chemotaxis. An initial burst of GPCR-stimulated PI3Kγ-dependent $PI(3,4,5)P_3$ accumulation could activate GTP exchange factors of the Rho/Rac family of small GTPases via activation of the exchange factor P-Rex-1, which is simultaneously activated by $PI(3,4,5)P_3$ and βγ subunits. Motifs on the p85 subunit mediate activation of PI3Ks by the small GTPase Rac1, which promote further $PI(3,4,5)P_3$ accumulation. Red lines represent positive and blue lines represent inhibitory signalling pathways.

It is evident that co-ligation of the BCR and FcγRIIb receptor does not lead to the complete inhibition of phosphorylated PKB. Since SHIP catalyses the

dephosphorylation of $PI(3,4,5)P_3$ to generate $PI(3,4)P_2$ and PKB has been shown to bind $PI(3,4,5)P_3$ and $PI(3,4)P_2$ with equal affinities, the actions of this phosphatase would not reduce the entire pool of lipids capable of binding PKB. Thus, the actions of PTEN would likely have a greater negative impact on the activation of PKB than SHIP. Although the PH domain of PKB has been reported to have dual specificity for both $PI(3,4,5)P_3$ and $PI(3,4)P_2$ (402,403), there are substantial *in vitro* data suggesting that $PI(3,4)P_2$ has a higher affinity than $PI(3,4,5)P_3$ for PKB (404-406). The addition of di-C16-$PI(3,4)P_2$ to serum-starved NIH3T3 cells stimulates PKB autophosphorylation, while di-C16-$PI(3,4,5)P_3$ causes slight inhibition (404). Studies in platelets have also shown that PKB activation correlates with $PI(3,4)P_2$ rather than $PI(3,4,5)P_3$ production following thrombin stimulation (404), while integrin cross-linking has been reported to generate $PI(3,4)P_2$ but not $PI(3,4,5)P_3$, yet still results in PKB activation (407). More recently, it has been suggested that $PI(3,4)P_2$ is essential for phosphorylation of PKB at Ser^{473} (408). In contrast, a high ratio of $PI(3,4,5)P_3$ relative to $PI(3,4)_2$ has been demonstrated to favour PKB activation in T cells, given the robust membrane localisation of the PKB-PH domain in unstimulated Jurkats. Moreover, the expression of a constitutively active SHIP protein in Jurkat cells was shown to cause large increases in $PI(3,4)P_2$ levels that correlated with a reduction in Ser^{473} phosphorylation and relocalisation of GFP-tagged PKB PH domains from the plasma membrane to the cytosol (293). Thus, taken together, these studies demonstrate that the specificity of PKB for $PI(3,4)P_2$ and $PI(3,4,5)P_3$ remains unclear.

Three pathways have been described to explain the possible mechanism behind FcγRIIb-mediated inhibition of the MAP kinase ERK1/2. Firstly, Hashimoto *et al* (1998) (224) suggested that SHIP inhibits PLCγ-induced PKC activation by blocking the initial translocation of Btk to the membrane. Secondly, Tridandipani *et al* (1997) (225,389) proposed that SHIP in addition to its catalytic activity, functions as an adapter. $p62^{dok}$, a Ras GAP-associated protein, is highly tyrosine phosphorylated in a Lyn-dependent manner upon crosslinking of the BCR with FcγRIIB (409). SHIP functions as an adaptor, binding Shc and $p62^{dok}$. The interation between $p62^{dok}$ and SHIP is mediated by the PTB domain of $p62^{dok}$ and requires tyrosine phosphorylation of SHIP, suggesting that it is mediated by phosphorylated NPXY motifs in SHIP. The binding of $p62^{dok}$ to RasGAP is thought to inhibit the Raf/MEK/ERK pathway through inactivation of Ras (227). In addition, the association of SHIP with Shc reduces the interaction of Shc-Grb2 and attenuates the activation of Ras, Raf and MAPK induced by positive BCR signalling (389,410). These results show that SHIP inhibits cellular activation through competitive binding (Figure 43).

The mechanism whereby SHIP inhibits chemokine-mediated ERK1/2 phosphorylation may also involve SHIP competing with Grb2 for the binding of Shc (Figure 44). Several non-receptor tyrosine kinases (NRTKs) and receptor tyrosine kinases (RTKs) have been proposed to participate in the pathway linking GPCRs to Ras-MAPK (411). Src or Src-like kinases can mediate the phosphorylation of Shc provoked by β-adrenoceptors and βγ-subunits (412). In this case, Src stimulation

appears to require the recruitment of β-arrestin to GPCR kinase 2 (GRK2)-phosphorylated β-adrenoceptors, followed by receptor internalisation and the consequent formation of molecular complexes between β-arrestin and Src (413). Alternatively, Src can be activated by direct interaction with $G\alpha_i$ and $G\alpha_s$ (414) or β_3-adrenoceptors (415). Therefore it is possible that FcγRIIb-mediated recruitment of SHIP could block the formation of Shc/Grb2/Sos complexes induced by antigen as well as chemokines.

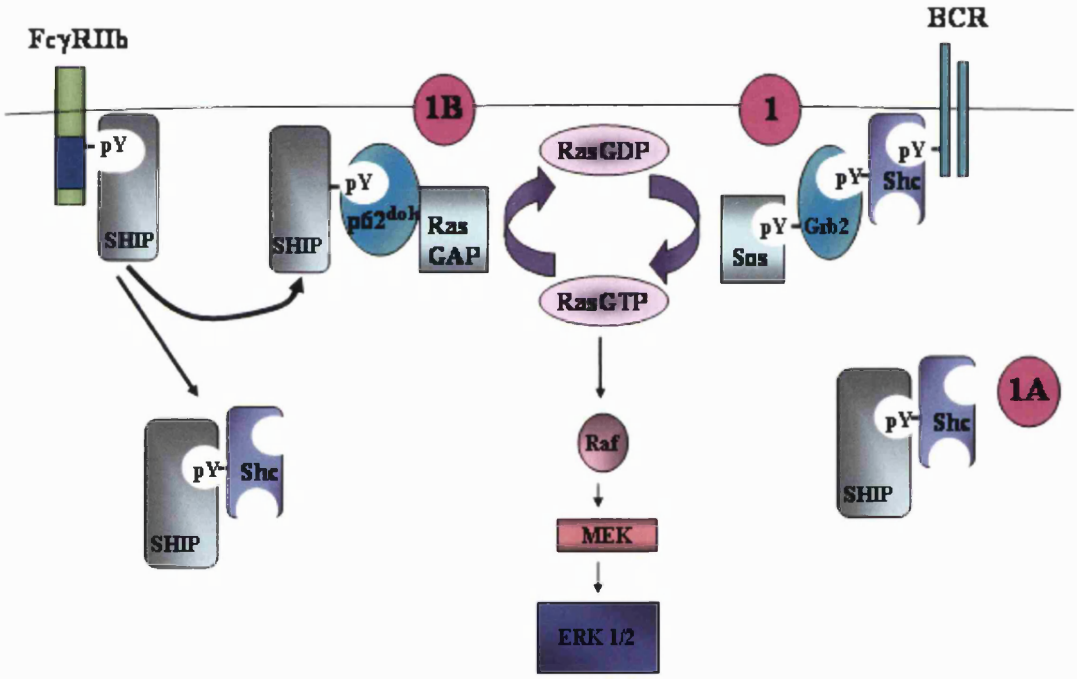

Figure 43. A Diagram High-lighting the Adaptor Function of SHIP in the Inhibitory Regulation of BCR-mediated ERK1/2 Phosphorylation

Stimulation of the BCR with antigen leads to activation of the ERK1/2/MAPK pathway via assembly of the Shc/Grb2/Sos complex (1). Two mechanisms involving the adaptor function of SHIP have been proposed to inhibit the ERK1/2/MAPK pathway: SHIP competes with Grb2 for binding to Shc and blocks the formation of the Shc/Grb2/Sos complex (1A); or SHIP is recruited to the FcγRIIb receptor and binds the RasGAP docking protein $p62^{dok}$, inhibiting the ERK1/2/MAPK pathway through inactivation of Ras (1B).

5.3 Concluding Remarks

The expression of chemokine receptors on lymphoid cells helps to guide the movements of re-circulating lymphocytes within lymphoid tissues (11,416). These 'lymphoid' or 'homeostatic' chemokines include CXCL12/SDF1, CXCL13/BLC, CCL19/ELC and CCL21/SLC. CXCR5 directs B cells to the follicle within a secondary lymphoid organ (spleen, lymph nodes, Peyer's patches and tonsil) where they can encounter antigen (138,417). The antigen-engaged B cell up-regulates the expression of CCR7, guiding its movements to the boundary of the follicle and T cell zone, where it receives T cell help (122,418,419). The B cells then undergo proliferation and many of the dividing cells begin making secretory immunoglobulin and become plasmablasts (420). This process is paralleled with a decreased responsiveness to both CXCR5 and CCR7 and increased responsiveness to CXCR4 (421,422).

Some plasma cell precursors, directed by CXCR4, leave the spleen and migrate to the bone marrow via the blood. In the lymph node, after activation and differentiation, plasmablasts travel to the medullary cords in a partially CXCR4-dependent manner. In this compartment they may terminally differentiate to plasma cells or pass into medullary sinuses to exit from the lymph node via the lymph. Carried into circulation, IgM or IgG plasma cell precursors may home to the bone marrow in a CXCR4-dependent manner (386). Thus, CXCR4 is not only required for plasmablasts

to migrate from the junction zones into the red pulp during terminal differentiation to plasma cells but also for the homing of plasma cells to the bone marrow.

In the setting described above, co-ligation of the BCR and FcγRIIb may not only function to reduce antibody production but also inhibit CXCR4-mediated trafficking of antibody secreting cells.

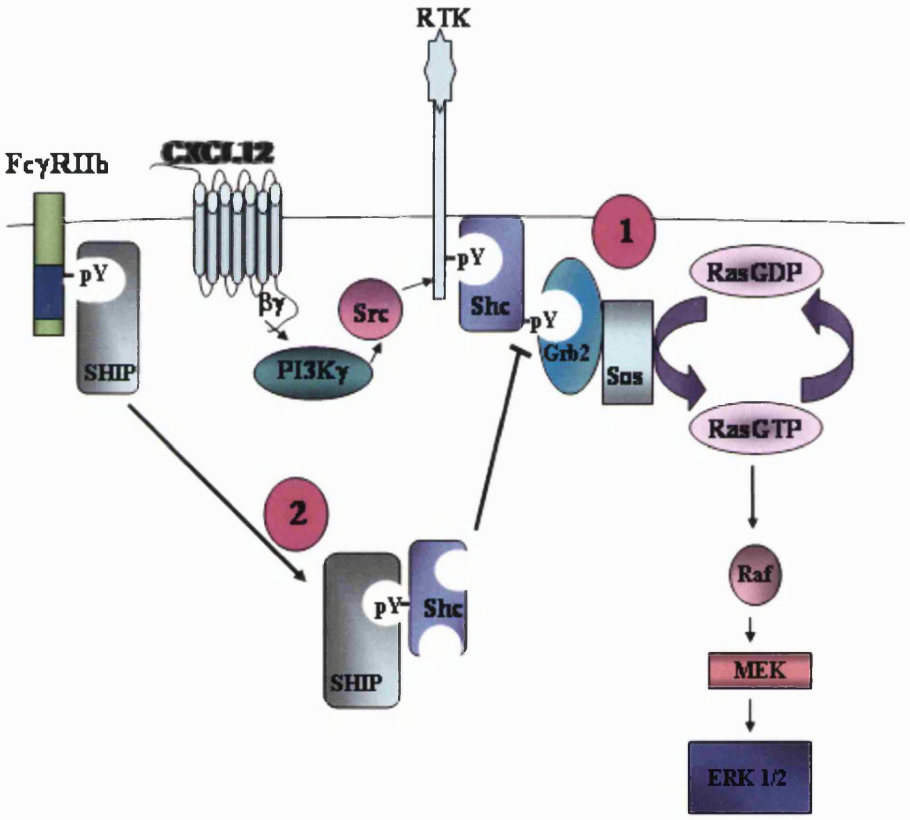

Figure 44. A Diagram High-lighting the Adaptor Function of SHIP in the Inhibitory Regulation of CXCL12-mediated ERK1/2 Phosphorylation

$G_{\beta\gamma}$ subunits have been shown to induce ligand-independent tyrosine phosphorylation of RTKs leading to activation of the ERK1/2/MAPK pathway via assembly of the Shc/Grb2/Sos complex (1). Co-ligation of the BCR and FcγRIIb results in the recruitment of SHIP which has been shown to compete with Grb2 for the binding of Shc. It is possible that FcγRIIb-mediated recruitment of SHIP could block the formation of Shc/Grb2/Sos complexes induced by antigen as well as chemokines (2).

Chapter 6

Chapter 6

Results: The Importance of PI3K-C2β in CXCR4 Internalisation

6.1 Introduction

At present, much of the information concerning the molecular mechanisms for GPCR sequestration and down-regulation is derived from the studies of β_2-adrenergic receptors (β_2-AR). This system has demonstrated the importance of adaptor proteins such as clathrin, β-arrestin and adaptin-2 (AP2) and enzymes such as Rab GTPases and PI3Ks in the regulation of signalling and internalization. Due to the large number of GPCRs and the diverse cellular function evoked by each individual receptor, it is conceivable that multiple mechanisms may be employed. Research into the regulation of chemokine-mediated responses has focussed primarily on CXCR2 (423-426) and CXCR4 (259,289,427-429).

Like most GPCRs, chemokine receptors undergo rapid desensitisation, internalisation and recycling after engaging with their ligands (428,430-433). This is believed to be an important mechanism by which inflammatory processes and HIV-1 infection are controlled. Many chemokine receptors internalise through phosphorylation-dependent processes involving the binding of β-arrestins to the receptors that have been phosphorylated by GRKs (259,426,434-438). A C-terminal serine-rich domain is required in CXCR4 for ligand-induced endocytosis of CXCR4 (438). β-arrestins

serve as adaptor proteins specifically targeting agonist-occupied receptors to clathrin-coated vesicles. A number of proteins involved in plasma membrane clathrin-coated pit function, such as AP2 and dynamin, are known to interact with phosphoinositides (439). Given that 3-phosphoinositides direct an array of molecules to different positions within the cell in response to different stimuli, some degree of spatial and temporal regulation of 3-phosphoinositide production must exist.

Whilst class I PI3Ks have been shown to interact directly with β-adrenergic receptor-kinase and $PI(3,4,5)P_3$ production is required for β-adrenoreceptor endocytosis, to date a role for class I PI3K in chemokine receptor internalisation/recycling has yet to be determined (440,441). Class II PI3Ks are thought to regulate clathrin-mediated membrane trafficking, where clathrin functions as an adaptor for PI3K-C2α (285). In addition, PI3K-C2α has been shown to be concentrated in the trans-Golgi network and present in clathrin-coated vesicles (285,286). Based on this observation, Class II PI3Ks may play a role in receptor internalisation/desensitisation and/or recycling to the plasma membrane, however the role of PI3K-C2β has not been examined. In conjunction with Prof. Ann Richmond (Vanderbilt University, Nashville USA), we investigated the subcellular localisation of PI3K-C2β with the goal of determining whether this PI3K could play a role in chemokine receptor internalisation/recycling. The human embryonic kidney cell line, HEK-293, was used in this section of the study because these cells do not endogenously express chemokine receptors and are easily transfected.

Generation of an EGFP-CXCR4 plasmid

To overcome initial methodological problems concerning the immunostaining of multiple proteins during receptor recycling, the first task was to generate an EGFP-CXCR4 construct. The problems that arose were not only down to the availability of antibodies but also due to the species in which they were derived. The aim was to perform triple subcellular staining visualisation experiments of CXCR4, PI3K-C2β and AP2. Since the commercial PI3K-C2β antibody was generated in a mouse, and the AP2 antibody was generated in a rabbit, the human CXCR4 antibody would have to be generated in a goat. Unfortunately, the mouse anti-goat secondary antibody required to detect the goat anti-human CXCR4 may cross-react with the primary mouse PI3K-C2β antibody, hence, the need to generate a fluorescent tagged CXCR4 construct. Using an EGFP-CXCR4 construct meant that the detection of CXCR4 would not require a fluorescent-tagged secondary antibody and thus permitting the straightforward detection of 3 different proteins at the same time.

The technique of polymerase chain reaction (PCR) was used to amplify the CXCR4 DNA sequence. Two primers (synthetic DNA oligonucleotides) were first designed, one complementary to each strand of the DNA double helix and lying on opposite sides of the region to be amplified. The PCR product was inserted into a pEGFP-N1 vector and cloned within competent cells. Following purification, the plasmid DNA was confirmed as EGFP-CXCR4 by two sets of restriction enzymes. The first cocktail contained *XhoI* and *HindIII* which cleaved the plasmid creating 2 bands

representing the 4.7 kb vector and the 1.1 kb CXCR4 insert. Many proteins are approximately 1 kb in size therefore an additional restriction digest was performed using *XhoI* and *BamHI*. The *BamHI* restriction site is not only present in the vector but also at 0.5 kb within the CXCR4 insert. *XhoI* and *BamHI* cut the vector at 3 points to produce a 0.64, 0.5 and 4.7 kb bands (Figure 45). The undigested band at 1.14 kb represents the CXCR4 insert plus the section of the vector up to the *BamHI* restriction site (refer to the Vector Map detailed in Chapter 3).

PI3K-C2β co-associates with the adaptor AP2

In eukaryotic cells, the formation and transport of vesicles from both the plasma membrane and trans-Golgi network (TGN) to endosomes involves the assembly of clathrin-coated vesicles (CCVs) (442,443). Polymerisation of clathrin with adaptor protein complexes in the cell is associated with phosphoinositide interactions and an array of receptors and regulatory molecules such as the GTPase ADP-ribosylation factor-1 (444). Tyrosine- and dileucine-based motifs represent two sorting signals contained within the cytoplasmic portion of several receptors and transmembrane glycoproteins. Such motifs mediate interactions with either the μ2 subunit of AP2 or the β1 subunit of AP1 to select proteins for inclusion into the vesicle and facilitate their transfer from one part of the cell to another (281,282). A long-standing question remains as to how the adaptor complexes can specifically target selected intracellular membranes. Since Class I PI3Ks have been shown to recruit AP2 to the receptor/β-

arrestin complex, adaptor complexes may interact with the D3-phosphoinositide products of PI3K isoforms or the enzymes themselves. Using a GST-AP2β fusion protein we first set out to investigate whether AP2 associates with the Class II PI3K-C2β *in vitro*.

GST-AP2β bound to glutathione beads were incubated with HEK-293 cells lysates. The proteins associating with the GST alone and GST-AP2 fusion protein were separated by SDS PAGE and immunostained with an antibody specific for Class II PI3K-C2β. A band corresponding to the molecular weight of PI3K-C2β (165kDa) was found to co-precipitate with the GST-AP2β fusion protein, but not with GST alone (Figure 46). Identical samples were immunostained with an antibody specific for GST, to verify the presence of the fusion protein and GST alone. The additional bands that were detected by the GST antibody may either be degradation products of the GST-AP2β fusion protein or alternatively a result of non-specific binding of the GST antibody.

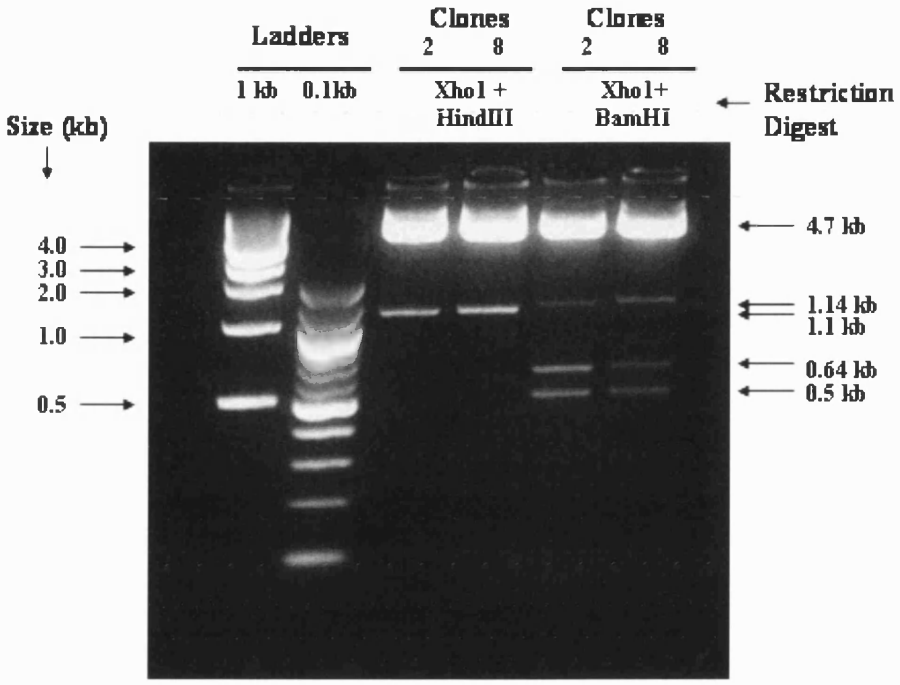

Figure 45. A restriction enzyme digest of the EGFP-CXCR4 plasmid.

Following a maxi prep of 2 positive clones (2 and 8) selected from 8 mini cultures, two separate digests were carried out to verify that the plasmid DNA was CXCR4. The first digest included the restriction enzymes *Xho1* and *HindIII* which resulted in the formation of 2 bands representing the vector and the insert CXCR4. The second digest included the restriction enzymes *XhoI* and *BamHI* which cut the vector in such a way that a 0.64, 0.5 and a 4.7 kb band should be produced if the insert has been introduced correctly. Refer to the vector map detailed in *Materials and Methods.* Data are from a single experiment representative of 3 others.

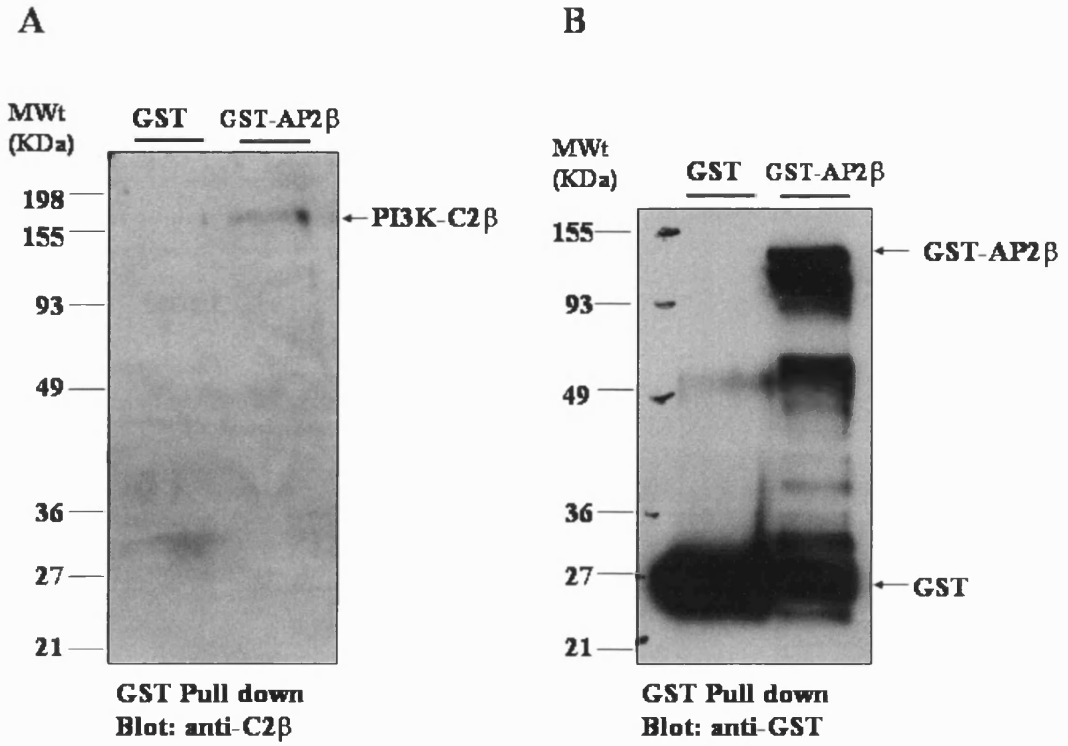

Figure 46. PI3K-C2β co-associates with GST-AP2β

Lysates derived from HEK-293 cells were incubated with glutathione-bound GST-AP2 fusion proteins overnight at 4°C. These data show western blot analysis of GST pull downs of HEK-293 cells using either an antibody specific for (A) murine PI3K-C2β; or (B) rabbit GST. Data are from a single experiment representative of 3 others.

The co-localisation of AP2 with GFP-CXCR4

β-arrestins have been shown to bind to clathrin with high affinity (445), although they are not constitutively associated with clathrin-coated vesicles (446), nor do they promote clathrin-coat assembly (447). These studies imply that other adaptor proteins are also involved in this process. In fact, recent studies on the β_2-adrenergic receptor (β_2-AR) have shown that adaptin 2 (AP2) functions as a clathrin adaptor for the endocytosis of β_2-AR (448). The role of the AP2 within chemokine-mediated receptor internalization is ill-understood, so experiments were designed to investigate the role of AP2 in CXCL12-mediated internalization of CXCR4. EGFP-CXCR4 transiently transfected HEK-293 cells were stimulated with CXCL12 (10 nM) over a time course manner of 0-30 minutes (Figure 47). Independently of agonist, EGFP-CXCR4 was consistently expressed at the plasma membrane 48 hr post-transfection. Following addition of CXCL12, EGFP-CXCR4 re-distributes from the membrane and appears in the cytosol with punctate distribution at approximately 5 minutes. The majority of the EGFP-CXCR4 receptor is internalised at 5 minutes but tends to re-appear at the membrane between 15 and 30 minutes. Cyanine 3-conjugated AP2 (red) has some constitutive membrane association but is also present at low levels in the cytoplasm. Upon stimulation of EGFP-CXCR4 with CXCL12, the localisation of AP2 with the plasma membrane is markedly reduced at 2 and 5 minutes post-stimulation, followed by an increase in cytoplasmic AP2 at 15 minutes. The constitutive AP2 is shown to co-localise with the membrane-bound EGFP-CXCR4 in

regions indicated by yellow colouring. Following stimulation of EGFP-CXCR4 with CXCL12, the co-localisation of AP2 with EGFP-CXCR4 is also evident in the cytoplasm (Figure 47).

The co-localisation of PI3K-C2β with AP2 and EGFP-CXCR4

Previously the Class II PI3K-C2α has been shown to be highly enriched in clathrin-coated vesicle fractions (449), to be constitutively associated with phospholipid membranes and disperse to perinuclear sites consistent with the ER. Immunofluorescence assays demonstrated that the distribution of PI3K-C2α within the perinuclear sites paralleled with that of γ-adaptin (AP-1) (285). Based on these observations, the role and distribution of PI3K-C2β was investigated. HEK-293 cells transiently transfected with EGFP-CXCR4 were stimulated for 5 minutes with 10 nM CXCL12 (Figure 48). The 5 minute time point was chosen as the optimal point at which CXCR4 was seen to internalise (Figure 47). The localisation of EGFP-CXCR4 changed in response to CXCL12 from the plasma membrane to a punctate distribution in the cytosol. Cyanine 3-conjugated PI3K-C2β (red) is shown to be predominantly and uniformly localised within the cytosol, however a punctate distribution can also be seen along the plasma membrane. Stimulation with CXCL12 leads to the re-localisation of cytoplasmic PI3K-C2β to perinuclear regions of the cell. Cyanine 5-conjugated AP2 follows a similar distribution to PI3K-C2β, where it is mainly distributed in the cytoplasm and re-distributes to regions adjacent to the

nucleus following stimulation with CXCL12. The overlay image shows that prior to stimulation with CXCL12, a small proportion of PI3K-C2β co-associates with EGFP-CXCR4 at the plasma membrane (yellow) whilst the majority co-associates with AP2 within the cytoplasm (pink). On addition of CXCL12, co-localised PI3K-C2β and AP2 redistributes to perinuclear regions of the cell. CXCL12-mediated internalisation of EGFP-CXCR4 co-localises with AP2 within the cytoplasm (pale blue) with some areas showing colocalisation of AP2, PI3K-C2β and EGFP-CXCR4 (white).

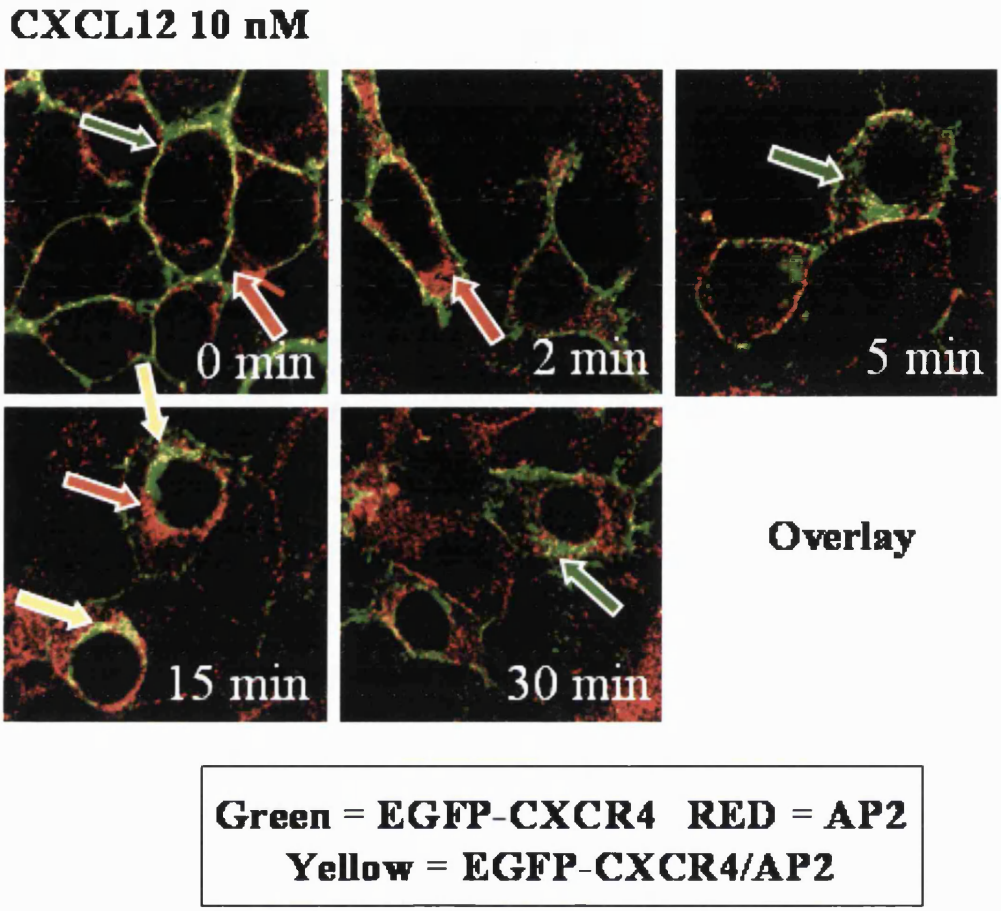

Figure 47. Co-localisation of EGFP-CXCR4 with mouse anti-AP2 in HEK-293 cells.

GFP-CXCR4-expressing HEK-293 cells were stimulated at intervals between 2 and 30 minutes with 10 nM CXCL12 and fixed with methanol. The cells were immunostained with a murine antibody specific for AP2. As described in *Materials and Methods* a cyanine 3 -conjugated secondary antibody was then used to detect AP2-β (red). Images of the cells were obtained by confocal microscopy. The coloured arrows indicate the areas of individual and co-localised proteins. Data are from a single experiment representative of 3 others.

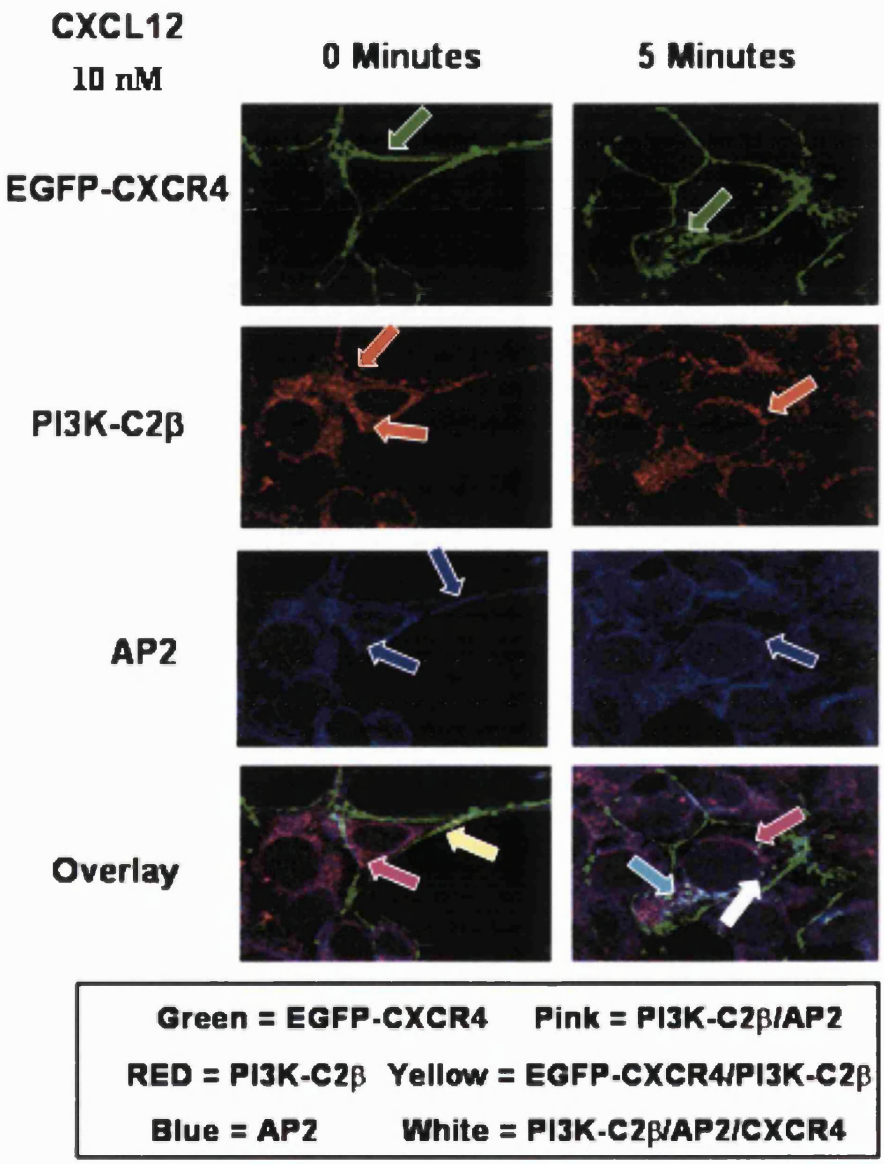

Figure 48. The co-localisation of PI3K-C2β with GFP-CXCR4 and AP2-β

HEK-293 cells, transiently transfected with GFP-CXCR4, were stimulated in a time dependent manner with 10 nM CXCL12 and fixed with methanol. The cells were immunostained with a cocktail of antibodies specific for PI3K-C2β and the adaptor protein AP2-β. Murine cyanine 3- and rabbit cyanine 5 – conjugated secondary antibodies were used to detect either PI3K-C2β or AP2-β respectively. Images of the cells were obtained by confocal microscopy. The coloured arrows indicate the areas of individual and co-localised proteins. Data are from a single experiment representative of 3 others.

The role of PI3K-C2β in the Recycling Endosome

Intracellular trafficking of membrane receptors is tightly regulated by a subfamily of ras-like small GTPases (Rab GTPases). Approximately 40 members of Rab GTPases have been identified, and each is believed to be specifically associated with a particular organelle or pathway. The recycling endosome is known to be regulated by Rab11a and Rab25 (450-452). Research into the family of Rab GTPases is in its infant stage therefore most members have yet to be investigated with regard to chemokine receptor trafficking. Since EGFP-CXCR4 appeared to recycle back to the plasma membrane around 15 minutes post stimulation with CXCL12 (Figure 47), we aimed to investigate the role of PI3K-C2β within the recycling endosome by focussing on the co-localisation with Rab11a (Figure 49). As seen in previous experiments, EGFP-CXCR4 is localised at the plasma membrane and in response to 10 nM of CXCL12, EGFP-CXCR4 internalises into the cytoplasm. The cytoplasmic distribution of cyanine 3-conjugated PI3K-C2β (red) and cyanine 5-conjugated Rab11a (blue), localises to perinuclear regions of the cell in response to CXCL12. The overlay image shows cytoplasmic co-association of PI3K-C2β with Rab11a (pink) and some co-association of PI3K-C2β with EGFP-CXCR4 at the plasma membrane (yellow). Following the addition of CXCL12, there appears to be an increase in the co-association of PI3K-C2β and Rab11a, as demonstrated by the enhanced pink colour. A small proportion of CXCL12-mediated internalised EGFP-CXCR4 is shown to co-localise with PI3K-C2β (yellow) and with both Rablla and PI3K-C2β (white).

Co-localisation of PI3K-C2β with phospho-PKB at the leading edge

Directional cell movement is known to involve the rapid activation of PI3K at the leading edge, leading to the production of $PI(3,4,5)P_3$ and $PI(3,4)P_2$, PH domain localization and movement up the chemoattractant concentration gradient (453). This part of the study focussed on the co-localisation of PI3K-C2β with the classical PH domain containing protein PKB in the presence of a chemokine gradient. Transiently transfected CXCR4 HEK-293 cells were presented with a gradient of CXCL12 for 5 minutes and fixed in methanol (Figure 50). Cyanine 3 (red) and cyanine 5 (green) conjugated secondary antibodies were used to detect the localisation of PI3K-C2β and phospho-thr^{308}-PKB respectively, in migrating CXCR4 expressing HEK-293 cells. PI3K-C2β (B) and phospho-thr^{308}-PKB (A) are predominantly distributed thoughout the cytoplasm however, a small proportion of these proteins are concentrated at leading edge of the cell in the direction of the chemokine gradient. The overview image (C) shows the co-localisation of these two proteins (yellow) which appears to be predominantly within the pseudopod.

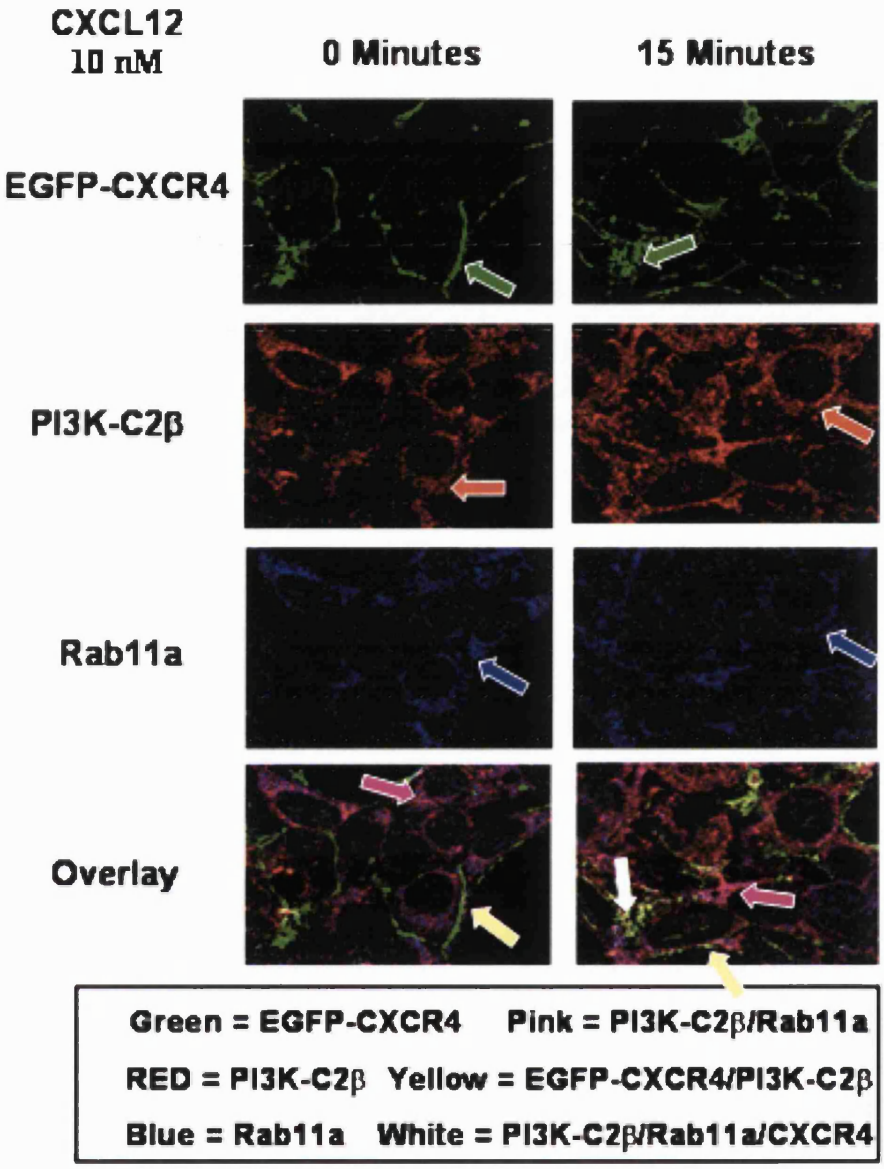

Figure 49. The co-localisation of PI3K-C2β with GFP-CXCR4 and Rab11a.

GFP-CXCR4-expressing HEK-293 cells were stimulated in a time dependent manner with 10 nM CXCL12 and fixed with methanol. The cells were immunostained with a cocktail of antibodies specific for PI3K-C2β and the enzyme Rab11a. Murine cyanine 3- and rabbit cyanine 5- conjugated secondary antibodies were used to detect either PI3K-C2β or Rab11a respectively. Images of the cells were obtained by confocal microscopy. The coloured arrows indicate the areas of individual and co-localised proteins. Data are from a single experiment representative of 3 others.

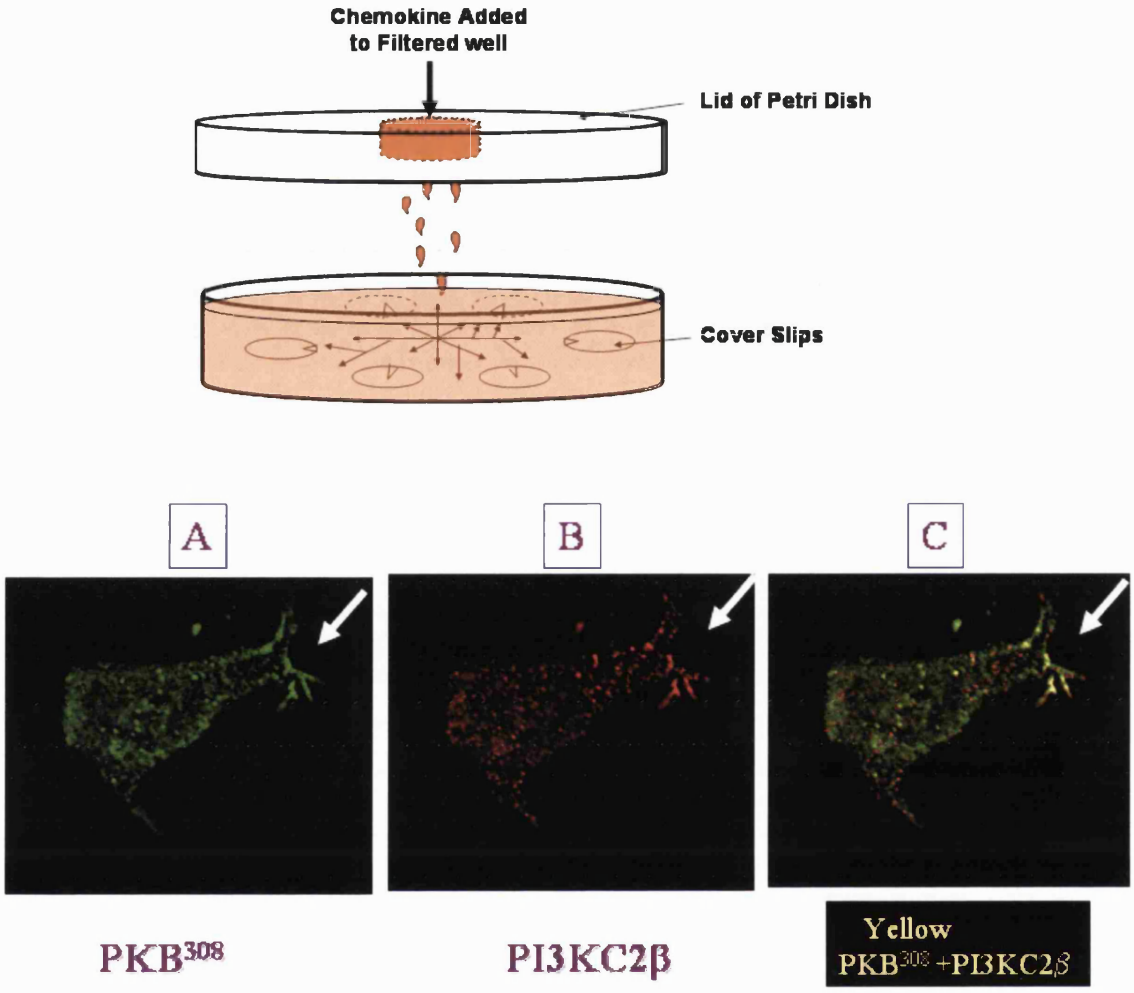

Figure 50. PI3KC2β Co-localises with phospho-thr^{308}-PKB at the Leading Edge. HEK-293 cells (5x10^5/ml) transiently transfected with CXCR4 were plated onto cover slips and left to adhere overnight in an incubator at 37°C. Cells were subsequently transiently transfected with CXCR4 and left to recover for 48 h. Cover slips were transferred into large petri dishes (See picture of apparatus above), stimulated with CXCL12 (10 nM) for 5 minutes and fixed in methanol. Cover slips were then incubated with a phospho-specific antibody to phospho-thr^{308}-PKB and PI3K-C2β for 30 minutes at RT. Following several washes with PBS, the cover slips were incubated with the murine cyanine 3- and rabbit cyanine 5- conjugated secondary antidbodies to detect either PI3K-C2β (B) or phospho-thr^{308}-PKB respectively (A). Finally the cover slips were mounted onto slides using an anti-fade mounting media and viewed on a semi-confocal/convoluting microscope. The white arrows indicate the areas of individual and co-localised proteins. Data are from a single experiment representative of 3 others.

Summary of Findings

- This study successfully designed, generated and expressed an EGFP-CXCR4 plasmid DNA construct.

- CXCL12 stimulated the internalisation of the EGFP-CXCR4 protein.

- PI3K-C2β co-localises with AP2-β in HEK-293 cells

- AP2-β is shown to constitutively co-localise with CXCR4 at the membrane.

- PI3K-C2β co-localises with AP2 in response to CXCL12.

- PI3K-C2β co-localises with phospho-thr^{308}-PKB at the leading edge of cells responding to a gradient of CXCL12.

6.2 Discussion

Phosphoinositides in Membrane Transport

Phosphoinositides are localised to the cytoplasmic face of cellular membranes where they can be substrates for numerous enzymes, including PI3Ks. These reactions rapidly and effectively alter the levels of particular phosphoinositides in specific regions of the membrane. There is a growing body of evidence indicating that changes in the phosphorylation state of the inositol ring of phosphoinositides regulate key steps in vesicular membrane traffic (454). $PI(4,5)P_2$, has been described as a key regulator of the formation, scission and uncoating of clathrin-coated vesicles (CCVs). Several proteins implicated in endocytosis, including epsin, CALM (clathrin assembly lymphoid myeloid leukemia protein) and AP180 (a brain specific CALM-homologue) bind specifically to $PI(4,5)P_2$. However, exactly how AP180 and epsin regulate the initial steps of clathrin-mediated endocytosis is not known, but it is likely that the interaction with $PI(4,5)P_2$ tethers the clathrin lattice to the plasma membrane (455). Other proteins involved in clathrin-mediated endocytosis have been found to bind to $PI(4,5)P_2$, including the GTPase dynamin, which is essential for scission of CCVs from the plasma membrane. Recent data show that both binding and hydrolysis of GTP by dynamin are required for the late stages of CCV formation (456).

The clathrin adaptor, AP2, is a target of 3-phosphoinositides, where the interaction with these lipids has been shown to regulate the recognition of endocytic signals and provide a distinct mechanism for increasing the specificity of sorting (457). The PI3K product PI(3)P has also been demonstrated to play a major role in endocytic trafficking. Studies employing a tandem FYVE domain as a probe for PI(3)P indicate that PI(3)P is highly enriched on early endosomes and in the internal vesicles of multivesicular endosomes and yeast vacuoles (458). Thus, it appears, the participation of specific phosphoinositides in membrane trafficking may reflect the ability of their phosphorylated inositol groups to bind specific proteins and protein modules.

Since membrane phosphoinositides can interact directly with molecules of the vesicle budding, docking and fusion machinery, cellular reactions that alter the levels of phosphoinositides might provide the fundamental matrix for the efficient regulation of vesicular traffic (459).

The Role of PI3Ks in Receptor Trafficking

The alteration of phosphoinositide levels by lipid kinase activity has been shown to be important for incorporation of mannose-6-phosphate receptors into clathrin-coated buds in TGN (460). Because a key enzyme for the generation of phospholipids is PI3K, it suggests a possible role for PI3K in the internalisation of receptors. The G-protein coupled receptor kinase, βARKinase1 has been shown to directly interact with

the PIK domain of PI3K to form a cytosolic complex. Overexpression of the PIK domain displaced endogenous PI3K from βARK1 and prevented βARK1-mediated translocation of PI3K to activated β_2ARs. Furthermore, disruption of the βARK1/PI3K interaction inhibited agonist-stimulated AP2 recruitment to the β_2AR and receptor endocytosis (280). Class I PI3Ks have also been shown to be required for the endocytosis of transferrin receptors which in this system occurs directly from the early endosome and independently of the recycling endosome (283).

Class II PI3Ks have also been shown to play a role in clathrin-mediated membrane trafficking. PI3K-C2α is reported to bind directly to clathrin, where clathrin stimulates the catalytic activity of PI3K-C2α, especially towards $PI(4,5)P_2$ (286). Endogenous PI3K-C2α is found in CCVs at the plasma membrane and in the TGN (285,286). Overexpression of PI3K-C2α inhibits clathrin-mediated endocytosis and sorting in the TGN (286). Thus, it is possible that the class II PI3Ks may contribute to the local phosphoinositide production in clathrin-coated pits, which may facilitate the recruitment of inositide binding proteins within the coat such as AP2, dynamin, synaptojanin and arrestin.

Agonist-induced endocytosis of most GPCRs occurs primarily via clathrin-coated vesicles. Previous studies have shown that CXCL12-mediated internalisation of CXCR4 involves GRKs, which function as adaptor molecules between the receptor and regulatory proteins such as β-arrestin and dynamin (254,259,428). The functions

of β-arrestins, dynamin and AP2β have all been found to involve binding to inositol lipids or inositol phosphates (461).

Since AP2 functions as an adaptor for clathrin and clathrin for PI3K-C2α, it is possible that class II PI3Ks participate in shared protein complexes with AP2. The data in this study revealed that a small proportion of AP2 constitutively co-localises with EGFP-CXCR4 at the plasma membrane. One possible explanation for this is the ability of the cell to mediate ligand-independent receptor recycling. This study also demonstrates that AP2 constitutively co-localises with PI3K-C2β in a uniform distribution within the cytoplasm, in CXCR4-expressing HEK-293 cells. Constitutive co-localisation of PI3K-C2β with AP2 suggests that PI3K-C2β may facilitate the recruitment of AP2 to the membrane where it is required for clathrin-coated pit formation. In response to CXCL12, the co-localised AP2 and PI3K-C2β appears to re-distribute to perinuclear regions of the cell. A similar distribution of PI3K-C2α and AP1 has been reported, where the perinuclear regions of the cell have been identified as the endoplasmic reticulum or Golgi apparatus (285). However, AP1 is present on vesicles derived from the Golgi apparatus, whereas AP2 is found on vesicles produced from the plasma membrane. In other studies, the perinuclear region of the cell has been described as the pericentriolar or perinuclear recycling compartment (PNRC) (462). This organelle is formed by tubulo-vesicular structures that gather recycling molecules directed to the plasma membrane once they have been separated from their cargo headed for the lysosomes (463). Since Rab11a, a small

GTP-binding protein, has been found mainly associated with the PNRC by Trischler *et al.* (464), we set out to determine if Rab11a co-associates with EGFP-CXCR4 and PI3K-C2β.

Rab11a and PI3K-C2β may regulate recycling of the chemokine receptor CXCR4

Recent studies have demonstrated that GPCRs are also found in perinuclear recycling endosomes during their intracellular trafficking steps (462,465). As mentioned previously, the recycling endosomes are distinguished from other endosomes by selective retention of recycling proteins and the presence of the small GTPase Rab11a (466,467). The cellular functions of Rab proteins are implemented by their ability to couple a structural GDP/GTP switch, common to all GTP-binding of the Ras superfamily, to a membrane/cytosol switch that depends on lipid modifications attached to their C-terminus, termed prenylation (468). In the simplest scheme, GDP-bound Rab proteins are maintained by GEFs. $PI(3,4,5)P_3$ directly and strongly activates the GEF activities of P-Rex1 and SWAP-70 through binding via PH domains (469). Thus, it is possible that the activation of GEFs required for Rab activation may also bind to PI3K-dependent D-3 phosphoinositides. Rab5 has previously been shown to activate both PI3K-dependent and PI3K-independent effectors that act in parallel to promote phagosome maturation (470), but the involvement of the class II PI3Ks was not investigated. Investigation of class II

PI3Ks within the recycling endosome showed that PI3K-C2β co-localised with Rab11a within the cytoplasm of EGFP-CXCR4-expressing HEK-293 cells. In addition a small proportion of internalised EGFP-CXCR4 co-localised with both PI3K-C2β and Rab11a following stimulation of cells with CXCL12.

Taken together these results suggest that CXCL12 induces the internalisation of CXCR4. We propose that PI3K-C2β contributes to local phosphoinositide production in growing clathrin-coated pits. AP2 may be recruited to the plasma membrane and bind PI3K-C2β-mediated D-3 phosphoinositide lipids and function as an adaptor for clathrin. These data also show that a proportion of internalised CXCR4 recycles back to the plasma membrane via the perinuclear recycling endosome, regulated by Rab11a. Activation of Rab11a may be dependent on the binding of GEFs to PI3K-C2β-mediated D3-phosphoinositides.

A role for Class II PI3K in CXCL12-mediated chemotaxis

One of the most important functions of chemokine receptors is receptor-mediated chemotaxis. Because inhibition of agonist-induced receptor endocytosis impairs the cell chemotaxis, it has been suggested that receptor internalization and recycling to the cell membrane may provide an on-off mechanism for the receptor-mediated chemotaxis or may be required for detection/response to the chemokine concentration gradient (426). Inhibition of agonist-induced receptor sequestration by mutation in

the carboxyl terminus of CXCR2 was shown to inhibit ligand-mediated chemotaxis of HEK-293 cells (423). During chemotaxis several studies have reported that PKB is selectively recruited via its PH domain at the leading edge of cells following chemoattractant stimuli (471). It is well-known that $PI(3,4,5)P_3$ is a major product of class IA PI3Ks that leads to the recruitment of PKB. Class II PI3Ks may also contribute to the phosphoinositide production at the leading edge of a cell, since in the presence phosphatidylserine, they can also phosphorylate $PI(4,5)P_2$. In addition, chemokines have been shown to activate class II PI3Ks in vitro which are thought to be responsible for mediating the *in vivo* formation of D-3 phosphoinositide lipids (197). Immunofluorescence analysis of fixed cells responding to a gradient of CXCL12 suggested that PKB^{308} co-localised with PI3K-C2β (yellow) at the leading edge of the cell in a time dependent manner. These data suggest that class II PI3Ks can contribute to phosphoinositide production and may also function to recruit PKB to the leading edge of chemotaxing cells.

6.3 Concluding Remarks

We propose that PI3K-C2β-mediated phosphoinositide production may be involved in the recruitment of AP2 to the CXCR4 receptor complex, where AP2 is likely to function as an adaptor for clathrin. Internalisation and subsequent recycling of CXCR4 appears to occur via the recycling endosome, regulated by Rab11a. PI3K-C2β-mediated phophoinositide production may be important for the binding and

activation of GEFs that are necessary for Rab11a activation. Finally, we show that PI3K-C2β localises to the leading edge of a migrating cell, where it appears to co-associate with PKB.

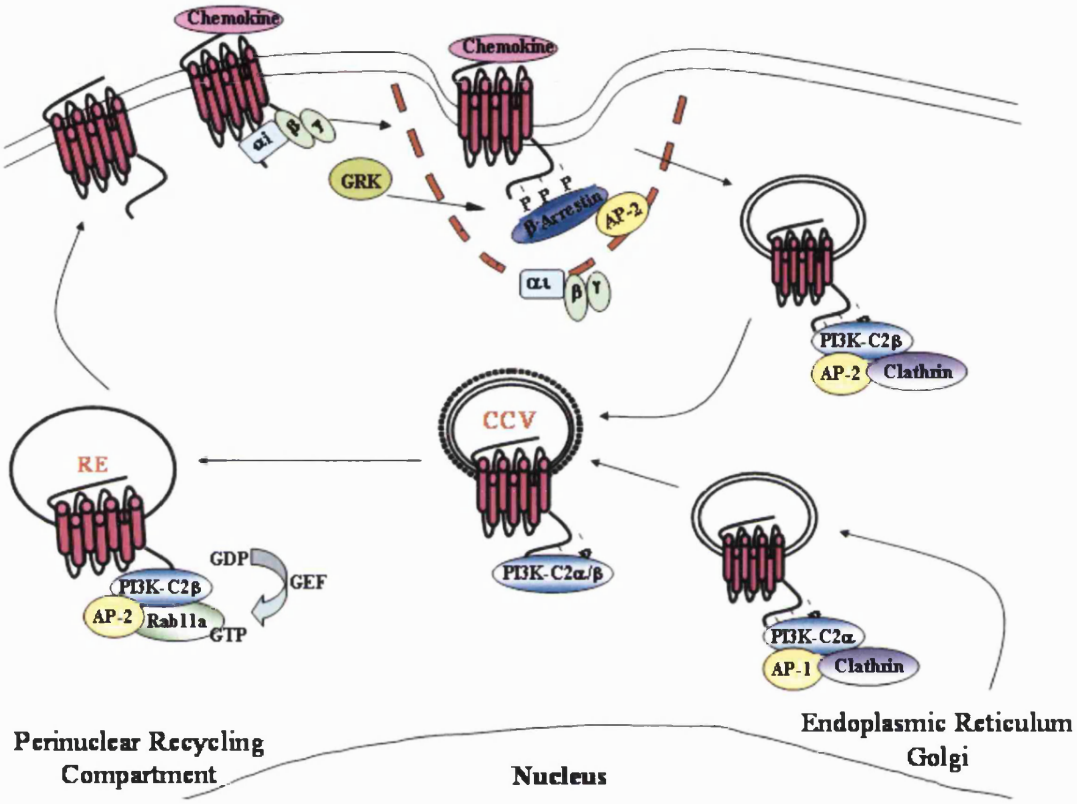

Figure 51. A Model Depicting the Function of Class II PI3Ks in Receptor-mediated Endocytosis

The mechanism of receptor cycling is a multi-step process involving the recruitment of many regulatory proteins and adaptor molecules, the majority of which bind to inositol lipids. The alteration of phosphoinositide levels by lipid kinase activity has been shown to be important for the incorporation of receptors into clathrin-coated buds. This diagram illustrates the areas in which we believe the class II PI3Ks are involved.

Chapter 7

Chapter 7

7.1 Overall Discussion and Future Directions

The signalling pathways that are mediated by chemokines and their respective receptors are complex and remain to be fully characterised. As described in the introduction the pathways stimulated by CCL1 have not been examined which provided an exciting window of opportunity in which to investigate. Using the CXCR4/CXCL12 receptor system as a model, the pathways identified following CCL1 stimulation have been shown in this study to be comparable. This does not imply that all chemokines elicit identical pathways because this is not the case. There appear to be similarities in the pathways that are mediated by different chemokines however it is evident that some chemokines may activate signalling pathways that others do not. Chemokine-mediated chemotaxis for example has been reported to be both PI3K-dependent (287) and independent (317). Studies of leukocytes from PI3Kγ knock-out mice reveal that PI3Kγ is involved in chemoattractant-mediated motility and cell migration (472,473), although neutrophils are reported to be dependent on PI3Kδ for the directional component of chemotaxis (333). Alternatively, signalling pathways may be cell specific, where certain cell types lack a particular pathway or protein. Taking this into account, data from studies using cell lines must be used as a guide. The Jurkat T cell line for example is known to lack both lipid phosphatases

SHIP and PTEN which would have dramatic effects on the constitutive levels of $PI(3,4,5)P_3$.

The data generated in this study have, for the first time, highlighted signalling pathways that are important for CCL1-mediated cell migration. Research in this field has shown that the binding of chemokines to their receptors is followed by the involvement of heterotrimeric G proteins, adenylyl cyclase, PLC, PLA, PLD, protein tyrosine and serine/threonine kinases, lipid kinases (PI3K), the Rho family of small GTPases (199,474), and the triggering of intracellular second messengers such as cAMP, phosphoinositides and calcium (77). Similarly, this study has demonstrated that CCL1-mediated migration of HUT-78 cells requires G proteins, PLC, PI3K, PKC and calcium. However, there remains a multitude of signalling molecules implicated in chemotaxis that have yet to be elucidated.

One of most impressive effects of chemokines on leukocytes are the morphological changes: the cytoskeleton is rearranged, integrin-mediated focal adhesions are formed, and the cell binds and detaches from the substrate in a coordinated manner with extension and retraction of pseudopods to execute the directional migration (475). The mechanisms thought to regulate the cytoskeletal rearrangements underlying leukocyte polarization and migration are a complex system of signal transduction molecules, including tyrosine kinases, lipid kinases, second messengers and members of the Rho family of small GTPases. Two members of the family of

small GTPases, Rac1 and Cdc42, signal the formation of lamellipodia and filopodia, resepectively (476). Potential pathways for the transduction of signals from active Rac and Cdc42 to actin polymerization into lamellipodia and filopodia have been uncovered (Figure 52). Of the many effector proteins that interact specifically with GTP-Cdc42, only the haematopoietic Wiskott-Aldrich-syndrome protein (WASP) and its ubiquitous family member N-WASP provide a direct link to actin assembly through activation of the nucleating activity of the Arp2/3 complex (477). Another signalling pathway implicated in lamellipodium formation involves the PAK protein family. These serine/threonine kinases were identified as direct downstream effectors of Rac and Cdc42. PAKs are engaged in multiple signalling pathways, some of which might be coupled directly to lamellipodium protrusion. For instance, PAK interaction with Cdc42/Rac increases the levels of phosphorylated MLC thought to be required for the anchorage of lamellipodia. In addition, PAKs were shown more recently to activate Lim kinases to phosphorylate and thereby block the severing/depolymerizing activity of cofilin, which is proposed to affect lamellipodium turnover (478).

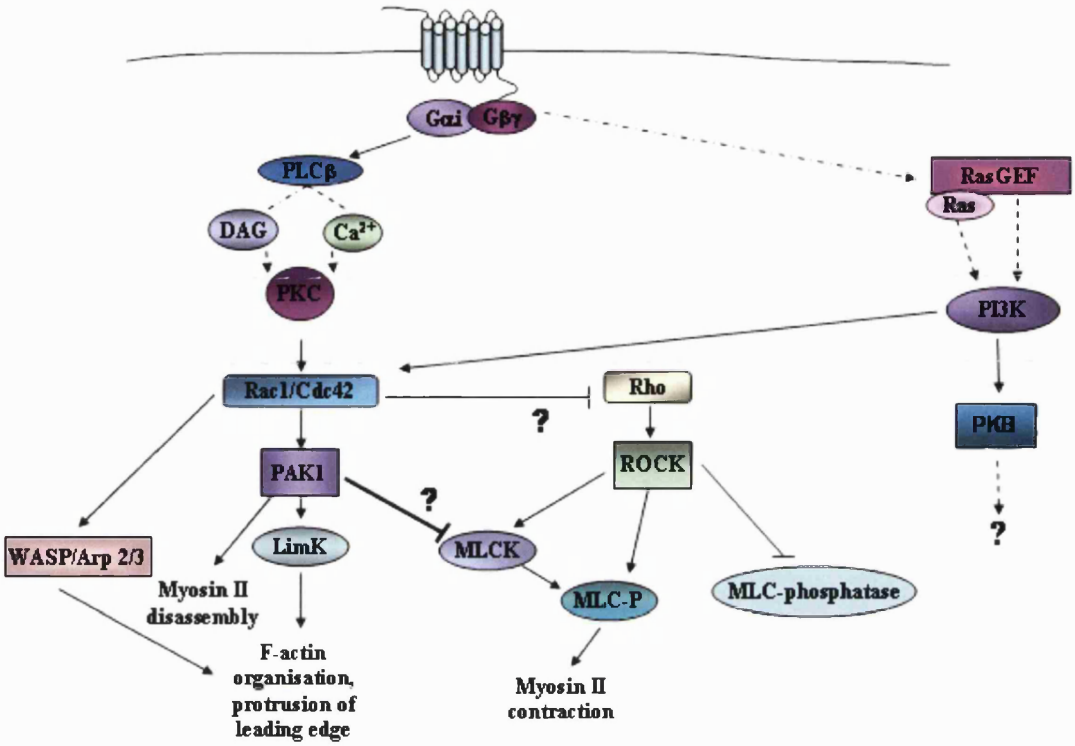

Figure 52 Signalling Pathways Implicated in Cell Migration

This figure illustrates the pathways that have been identified in this thesis and shows how they may fit in with the known mechanisms of cell migration.

Rho activity in migrating cells is associated with focal adhesion assembly and cell contractility, and is responsible for cell body contraction and rear end retraction. One important Rho target involved in stimulating actin: myosin filament assembly and therefore contractility is the Ser/Thr kinase p160ROCK. Rho and p160ROCK have been shown to be essential for detachment of the back of migrating cells such as leukocytes and macrophages (479,480). p160ROCK interacts with and phosphorylates the myosin binding subunit (MBS) of MLC phosphatase and thereby inactivates it (481). This leads to increased levels of myosin phosphorylation, which

then can cross-link actin filaments and generate contractile force. At the rear of a migrating cell, this promotes movement of the cell body and facilitates detachment of the cell rear (482). Clearly, Rho activity at the front of a migrating cell is incompatible with membrane protrusion and hence mechanisms must be in place to inhibit its activity at the leading edge. One way this might occur is through Rac. The biochemical mechanisms involved are not entirely clear; one suggestion is that p65PAK phosphorylates and inactivates MLCK, leading to decreased levels of myosin phosphorylation (483). However, others have reported that p65PAK can phosphorylate myosin light chain directly, thus enhancing cell contractility (484). Thus, Rho GTPases are key players in the process of cell migration and their ability to cycle between active and inactive states allows the cell to respond rapidly to extracellular cues.

Interestingly, ligand-stimulated receptor endocytosis has also been demonstrated to be essential for chemotaxis. Inhibition of agonist-induced receptor sequestration by mutation in the carboxyl terminus of CXCR2 was shown to inhibit ligand-mediated chemotaxis (423). Given that PI3Ks contribute to the level of phosphoinositides, which play a central role in receptor recycling this study investigated the role of the class II PI3K-C2β. The data revealed that PI3K-C2β appeared to be involved in the internalisation and recycling of CXCR4 and was also shown to be concentrated at the leading edge of a migrating cell.

PI3Ks and their lipid product $PI(3,4,5)P_3$ have been widely implicated in controlling cell migration and polarity (390). During leukocyte chemotaxis, type IA PI3Ks are required for lamellipodium extension and migration towards colony-stimulating factor 1 (485), whereas type IB PI3Ks are required for neutrophil chemotaxis to a variety of inflammatory mediators that signal via GPCRs (392,393). GFP-tagged PH domains have been used to visualize bursts of $PI(3,4,5)P_3$ induced in response to extracellular stimuli, and studies reported in *Dictyostelium*, neutrophils and fibroblasts reveal co-localisation with centers of actin polymerization at the front of the migrating cell (486-488). Rac activation has been shown to stimulate PI3K, leading to the accumulation of $PI(3,4,5)P_3$ and filamentous actin at the leading edge (184,475,486). Disruption of this positive feedback loop results in an erratic, nonpolarized cell response to chemoattractants (330,489).

Studies in *Dicytostelium* have shown that the localisation of PI3K at the front is coupled to a restricted distribution of its antagonist, the $PI(3,4,5)P_3$ phosphatase PTEN, at the rear and lateral edges, and that this is essential for chemotaxis (328,329,487). Since SHIP is also a $PI(3,4,5)P_3$ phosphatase that cleaves the 5' phosphate of $PI(3,4,5)P_3$ to generate $PI(3,4)P_2$, it is possible that SHIP may also function at the sides and back of the cell to reduce the levels of $PI(3,4,5)P_3$. The data presented in this study demonstrated that constitutively expressed SHIP led to the reduction of CXCL12-mediated cell migration, implying that a global reduction in $PI(3,4,5)P_3$ levels prevented the cell from polarizing and distinguishing front from

back. Alternatively, whilst PTEN functions to regulate the levels of $PI(3,4,5)P_3$ at the sides and back of the cell to promote forward movement, SHIP may be required at the leading edge to discontinue cell migration.

In summary, this study has focussed on three areas: signalling pathways; inhibitory regulation by the inositol-lipid phosphatase SHIP and receptor recycling, all of which, in some way or another appear to be involved in cell migration. Thus, despite the vast number of still remaining un-answered questions concerning the signalling pathways that regulate cell migration, this work has built the foundations upon which to further dissect the mechanism of signalling through chemokine receptors, these central mediators of leukocyte migration.

7.2 Future Directions

- The data presented within this study were generated using cell lines however, it is acknowledged that the use of transformed cell lines and expression of mutated proteins may not accurately reflect physiological events. Future experiments should be carried out using normal peripheral blood derived T cells.

- Once a functional human antibody specific for CCR8 is made available the immunofluorescence analysis of receptor endocytosis could be investigated. It

would be interesting to see if PI3K-C2β had an equivalent involvement in the regulation of CCR8 to that demonstrated with CXCR4.

- Further investigation into the regulation of CXCR4 sequestration would be of great interest. Future studies could investigate Hsc/Hsp70 Interacting Protein (Hip) which has recently been shown to associate with CXCR2 and regulate receptor signalling and trafficking. In addition, myosin Vb, a member of the class V unconventional myosins is reported to interact with Rab11a in the recycling of the transferrin receptor to the plasma membrane. Having demonstrated that CXCR4 co-associates with Rab11a, the next stage would be to see if myosin Vb was also involved.

- Further analysis is required to determine the exact role of PI3K-C2β in chemotaxis, whether it may be required for the production of lipid products or have some other unknown function. A possible approach may be to design imaging experiments to investigate the localisation of Class II PI3Ks with regulatory components.

- Having demonstrated that PI3K regulates CCL1-mediated chemotaxis of HUT-78, it would be interesting to determine which isoforms of PI3K are important. This could be achieved using chemically synthesised siRNA oligodeoxyribonucleotides to specifically inhibit the different isoforms.

Antisense agents are valuable tools to inhibit the expression of a target gene in a sequence-specific manner.

Appendix 1

Lysis Buffer (pH 7.5)		
		g/100ml
20mM Tris	(MW: 121.1)	0.242
137mM NaCl	(MW: 58.44)	0.801
10mM NaF	(MW: 41.99) 500mM stock	2 ml
1mM EDTA	(MW: 41.99) 100mM stock: 372.24mg/10ml	1 ml
10% glycerol	(w/v)	10.0
1% NP-40	(w/v)	1.0
1mM Sodium Orthovanadate	(MW: 183.9) 500mM stock	0.2 µl/ml
1mM PMSF	(MW: 174.2) 500mM stock in DMSO	2 µl/ml
Aprotinin	(2mg/ml) stock	2 µl/ml
Leupeptin	(2mg/ml) stock	2 µl/ml
Pepstatin	(5mg/ml) stock	2 µl/ml

Tris Buffered Saline (TBS)/Washing Buffer (pH 7.5)	
	g/l(10x stock)
10mM Tris	12.11
100mM NaCl	58.44
0.1% Tween-20 (TBST only)	10.0 ml

Buffer for Semi-Dry Transfer	**g/l**
48mM Tris	5.81
39mM Glycine	2.93
SDS	0.375
Methanol	200 ml

2x Sample Buffer (pH 6.8)	**g/100 ml**
125mM Tris	1.51
4% SDS	4.0
10% Mercaptoethanol (v/v)	10.0 ml
20% Glycerol (w/v)	20.0
0.04% Bromophenol Blue	0.04

4x Resolving Gel Buffer (pH 8.8)	
	g/500 ml
1.5M Tris	90.86
0.4% SDS	2.0

4x Stacking Gel Buffer (pH 6.8)	**g/500 ml**
0.5M Tris	30.29
0.4% SDS	2.0

SDS-PAGE Gel Solutions made with Resolving and Stacking Gel Buffers containing 0.4% SDS

Resolving Gel (20 ml)								Stacking Gel (12ml)
	5%	7.50%	10%	12%	14%	15%		5.00%
dH_20	11.51 ml	9.84 ml	8.17 ml	6.84 ml	5.51 ml	4.84 ml		6.85 ml
Resolving Gel Buffer (pH8.8)	5.0 ml	5.0 ml	5.0 ml	5.0 ml	5.0 ml	5.0 ml	Stacking Gel Buffer (pH 6.8)	3.0 ml
Bis-Acryl (30%)	3.33 ml	5.0 ml	6.67 ml	8.0 ml	9.33 ml	10.0 ml		2.0 ml
10% AMPS	150 µl	150 µl	150 µl	150 µl	150 µl	150 µl		150 µl
TEMED	15 µl	15 µl	15 µl	15 µl	15 µl	15 µl		10-15 µl

PCR – Master Mix	
QIAGEN Taq DNA Polymerase Kit	
10X Expand Buffer	5 µl
dNTP (10 mM)	0.4 µl
3' (25 pM)	1 µl
5' (25 pM)	1 µl
$MgCl_2$ (25 mM)	1.5 µl
Taq DNA Polymerase	0.2 µl
hCXCR4 Template cDNA* (0.1 µg/ml)	2 µl
dH_20	39 µl
TOTAL	**50 µl**

*** Template cDNA of human CXCR4 was previously sequenced by Prof. Ann Richmond from Vanderbilt University and used in this PCR master mix.**

PCR-2% Agarose Gel in 0.5x TBE	
Agarose Powder	4 g
1x TBE	200 ml
Ethidium Bromide	40 µl

Digestion Mixture		
PCR Product		**EGFPN1 Vector**
2 µl	Restriction Enzyme Buffer B (Promega)	2 µl
1 µl	XhoI (10 Units/µl)	1 µl
1 µl	HindIII (10 Units/µl)	1 µl
16 µl	PCR/EGFPN1	6 µl
0 µl	dH_2O	10 µl
20 µl	**TOTAL**	**20 µl**

PCR-10x TBE Stock	
	per 1 L in MilliQ
Tris Base	108g
Boric Acid	55g
0.5M EDTA (pH 8)	20 ml

PCR-Blue Juice
Bromphenol Blue 0.25%
Ficoll (Type 400) in Water 15%

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